BACTERIOLOGICAL TECHNIQUE

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A GUIDE FOR MEDICAL LABORATORY TECHNICIANS

BY

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WITH A FOREWORD BY

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"TO DOT"

As a small token of my appreciation for your help in checking the script and proofs, and especially for your tolerance of a very pre-occupied husband during many months past.

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FOREWORD

THE distribution of work in a clinical pathological laboratory has changed very much in the last quarter of a century. Many routine examinations which used to be done by the pathologist are now done by technicians. These technicians have to be trained.

In the past laboratory attendants gradually picked up certain technical methods, but there was no systematic instruction. The I.M.L.T. has changed all this. By the institution of courses of instruction, an intermediate examination for the young student technician and a final examination in one of the branches of pathology, they have materially raised the standard of the technician. The result is that anyone who is an Associate of the I.M.L.T. should have a considerable knowledge of the technical methods of his own special branch of pathology as well as some knowledge of the general methods used in all branches. But we are short of books for the student technician to read. Mackie and McCartney's *Practical Bacteriology* is admirable, but it is written for the medical student who has an outlook quite different from the student technician. Such a book obviously cannot go into detail of the elementary methods of laboratory technology.

A book was needed which gave precise instructions in such elementary details. Such a book could only be written by an experienced technician and it is with the intention of filling the gap that the author has produced this volume.

The reviewers will praise or criticize the details of the book, but I have had the opportunity of reading the proofs and I am sure that the author has done a great service by writing a volume which was sorely needed by the student technician.

ALEXANDER FLEMING.

PREFACE

THIS volume must not be regarded as a textbook on Bacteriology. It is intended, as its title implies, to be a guide for those who have chosen Bacteriological Technology as a career and deals with this subject from a technological viewpoint. Appreciation and understanding of the role which bacteria play in the "scheme of things" is best obtained by a study of books dealing specifically with Bacteriology.

The author has based much of the material within these pages upon his past twenty-five years' experience in various hospital and public health laboratories and, of recent years, in a large teaching establishment.

The accumulation of notes during and after training seldom, if ever, includes the sources of information, so that the author must of necessity express his thanks generally for ideas which cannot be individually acknowledged; he is deeply indebted to all who have offered suggestions and constructive criticisms during the preparation of the manuscript.

The author expresses here his deep gratitude to Professor Sir Alexander Fleming for his great kindness in writing the Foreword; to his friends and late colleagues, Eric G. Knowles for the special chapter on the Microscope, and John W. Gailer for his help with notes on the pH meter and its application. He extends his thanks to Lt.-Col. J. H. Bensted, R.A.M.C. (Retd.) for his encouragement and help during the early stages of preparing the manuscript.

Thanks are also due to Messrs. E. W. Taylor, L. C. Martin and B. K. Johnson for their kindness in permitting the use of illustrations from their respective publications and to The Royal Society; Messrs. Blackie and Son; The Hatton Press Ltd.; Messrs. E. & S. Livingstone Ltd.; Messrs. A. Hallenkamp & Co., Ltd., and The Berkefeld Filters and Water-softeners Ltd., for permission to reproduce illustrations and excerpts acknowledged individually in the text.

W. W. McEWEN.

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CHAPTER I

LABORATORY ACCOMMODATION AND EQUIPMENT

So much depends upon the premises available and the amount and variety of work undertaken that it would be impossible to lay down any hard and fast plans for accommodating the various activities which are involved in Medical Laboratory work. In any case, in most laboratories Bacteriology is but one section of the work involved and provision may have to be considered for separate premises for Pathology, Chemical pathology and Haematology. We will confine ourselves here to the requirements of an average laboratory and the following must be considered as a general guide only.

There must be:

(*a*) A cleaning and preparation room, (*b*) Director's and General laboratories, (*c*) Media room, (*d*) Animal house and, of course, Office premises (with which we need scarcely concern ourselves here!)

The Cleaning and Preparation Room

Regarding the structure of the cleaning room, simplicity of design with a minimum of corners and ledges is to be aimed at. As stated above, so much depends upon the premises allocated for the purpose, amount of work to be done, and possibility of converting such premises. Nevertheless, walls should be either of white tiles or sound gloss-paint or enamel. Floors should be of unbroken concrete or "terrazzo". If the floors are of wood, it is better to keep them bare and polished than to cover them with linoleum. Continual walking will cause linoleum to stretch slightly when it will act rather like a pair of bellows—each time a weight is put on it the dust underneath will be blown out into the atmosphere of the room.

The task of maintaining a regular supply of reliable apparatus and equipment for use in the laboratory is a very important one and, like all responsible tasks, cannot be done efficiently in unsuitable premises, with inadequate tools or without plenty of bench-space to set out the work.

The first consideration is plenty of running water—both hot and cold—and large medium-deep sinks. Next a series of vessels suitable for complete immersion of the various kinds of laboratory glassware are required and for this large earthenware bowls (with the interior surfaces glazed) are best. These bowls should be set up in a row on a low bench adjoining the sink and should be clearly labelled as to their contents. One containing 5 per cent. lysol, one containing strong hydrochloric acid and a third containing weak potassium permanganate solution. The bowl containing the acid should have a well-fitting lid to prevent acid fumes escaping to become a source of irritation to the workers. The bench should be sufficiently low to bring the rims of the bowls level with the top of the sink. Hanging on hooks over each bowl should be large loose-fitting rubber gloves (post-mortem gloves with long gauntlets reaching to the elbow of the wearer).

The sink (or sinks) should be equipped with both ordinary water-taps and long curved narrow taps (swan-neck) for obtaining fine jets of water under pressure (see Fig. 1). Also, it is better to have large enamelled bowls in the sinks than to fit the sinks with a plug in the waste-pipe. At the side of the sink, over a well-sloped draining board, a series of pegs should be fitted for draining vessels which have no "shoulders" such as conical flasks, cylinders, tubes, etc. For draining vessels with shoulders a horizontal rack consisting of struts of wood at varying distance apart may be fixed beside the peg-rack (Fig. 2). A number of heavy-gauge galvanized wire baskets for draining test-tubes should be available on the draining board. A long narrow double rack, with small holes in the top layer and the lower part made of fine-mesh galvanized wire netting or perforated zinc sheet may be fitted along the wall at the back of the sink, for draining pipettes of various sizes and shapes. On a lower shelf at the back of the bench adjoining the sink, winchesters of: 10 per cent. potassium dichromate, commercial grades of sulphuric and hydrochloric acids, pure lysol and a saturated solution of potassium

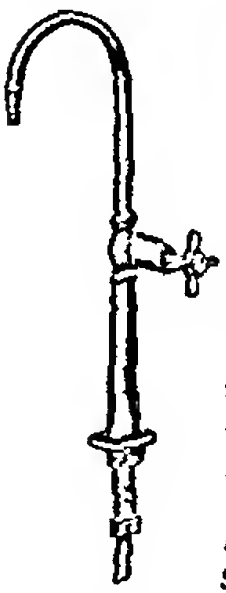


Fig. 1
Swan-neck tap

permanganate may be kept. (NOTE.—All containers used for storing cleaning fluids must be clearly labelled "FOR CLEANING ONLY".)

Away from the sink, a small section of a bench (of normal height) may be set out for dealing with microscope slides. The equipment here should include a small gas ring-burner, a heavy-gauge enamelled iron dish, three shallow wide-mouthed

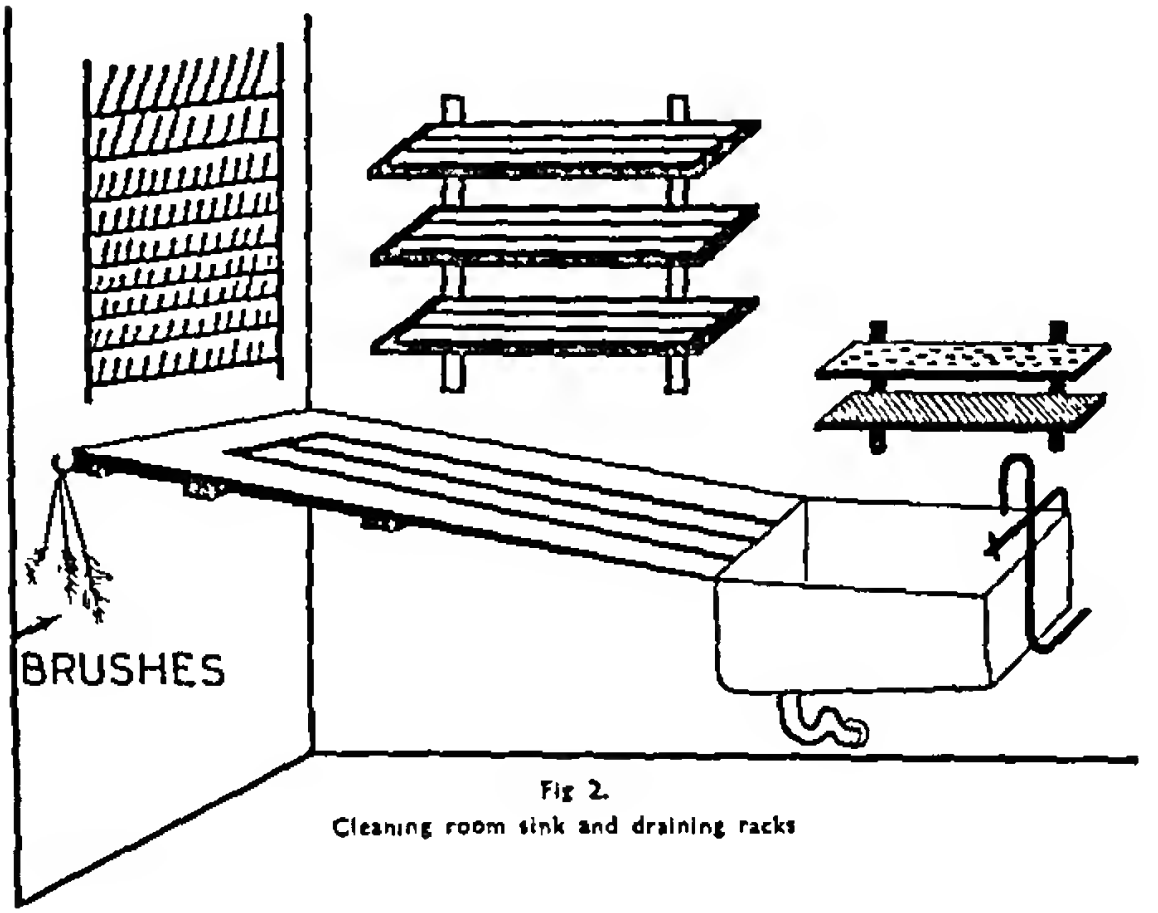


Fig 2.
Cleaning room sink and draining racks

stoppered jars (one containing methylated spirit, another xylol and the third empty), a small hot-air oven (preferably made of sheet asbestos, but with asbestos shelves anyway—such an oven if not already available can be readily improvised in most laboratories from the interior of an old incubator or similar metal structure from the "lumber-room"), and a small drawer very strictly reserved for grease-free glass-cloths which should be frequently laundered and kept free from starch.

Yet another section of the bench may be fitted for plugging, sorting and labelling. On shelves at the back of this part

of the bench, plainly labelled boxes can be kept into which are placed the various-sized corks, screw-caps, liners for screw-caps, etc., as they are released from the cleaning process or from stock. Labels, each size in its own box, may also be kept on these shelves. Incidentally, it requires but little ingenuity to devise a series of pigeon-holes of widths to fit the various labels employed, remembering that they should be tilted slightly backwards to prevent the labels falling out over the bench.

Also on the shelves at this part of the bench can be kept the various cotton-wools used for plugging, swab-making, etc. For ordinary routine plugging of tubes, neck-wool as used in barbers' saloons is undoubtedly the best. If this is kept in wooden boxes, with a small hole in the lids and lying on their sides with the holes facing towards the bench front, the wool can be used through the holes economically and will be kept clean throughout the life of each roll.

It seems to be common practice for the cleaning-room staff to be responsible for maintaining the supply of distilled water for the laboratory. In this case suitable accommodation, providing water-supply and a run-away for the cooling system must be given consideration when planning the cleaning-room.

The Media-Preparation Room

Wherever possible the room used for the preparation of culture-media (or for storing it in bulk) should be reserved and equipped exclusively for this purpose. Except perhaps for the general sterilization of apparatus, no other type of work should be carried out in the media-room. The room should be free from draughts, well lit and with as few exposed shelves and other possible dust-traps as possible. White tiled walls with rounded corners is the ideal, of course, but this is not always practicable; in which case the walls should be painted with an enamel finish. Plenty of bench-space should be available, the tops of the benches being either highly polished wood or linoleum (which should be glued to the bench over the entire surface). At least one large sink with hot and cold water, a draining board and racks similar to those in the cleaning

room, and, at the back of the sink, a long rack for the various pipettes in use. (It is strongly recommended that this rack be fitted over the sink as drippings from pipettes allowed to remain on the bench or floor can prove a source of contamination.) It is difficult to indicate a complete list of apparatus and equipment for the media-room as much depends upon the scope of the laboratory concerned. The room should, however, contain: an Autoclave, Koch (or Arnold) steamer, inspissator, large gas-ring, an enamelled-iron (or aluminium) saucepan to hold at least five litres, a pH comparator (Lovibond or—if very fortunate!—pH meter), a number of stout wire baskets (5 in. × 4 in. × 3 in. is a good size to use as tubes will not fall sufficiently sideways to become wedged) and a bunsen burner. Various types of bacterial filters should be available, the choice of which will depend upon the character of the work undertaken. Other equipment, apparatus and reagents will be indicated by the type of work carried out in the laboratory, the amount of media used and the rate at which it is consumed. Storage accommodation should be in the form of large shallow cupboards with well-fitting glass-panelled doors.

Work-room (or Laboratory)

When large numbers of certain types of specimens have to be examined regularly, as so frequently happens in the laboratories of Public Health Departments and large institutions, it is necessary for the work to be carefully planned and for adequate bench-space to be available in order that reports may be given in reasonable time. It is not only a waste of time and effort, but inviting errors to have to clear a bench each time a fresh task is undertaken. Again, the work of individual laboratories varies enormously and the best policy here is, perhaps, to give an example of what could reasonably be expected to constitute an average week's work for a senior technician at, say, a Public Health Department laboratory serving a large city or town. Let us say that he is responsible for routine bacteriological examinations coupled with a small miscellany of odd specimens involving haematological, pathological and path-chemical work.

During the course of an average week he is responsible, say, for the examination of:

Sputa (for M. tuberculosis)	100 to 150
Throat-swabs (for C. Diphth.)	100 to 150
Stools (for culture)	15 to 20
Miscellaneous (pus, C.S.Fs., pleural effusions, etc.)	0 to 10
Urines (for culture)	0 to 20
Widals	0 to 20
Blood counts	0 to 10
Path. chem. and sections, etc	Occasional

Such a programme of work would require at least 300 to 350 square feet of floor space (either a room to himself or part of a large room). At least 50 square feet of bench space (the bench to be not more than 2 ft. 6 in. wide) with shelving above the bench, a good-sized shallow cupboard and, if not a refrigerator to himself, at least a liberal share in a large "community" one. It is best to allocate sections of bench-space to specific kinds of specimens and to equip these accordingly. Where large numbers of any particular type of specimen are received regularly, sections of the bench should be laid out systematically for these, leaving one section of the bench clear for work that is received only occasionally, and which can be cleared rapidly to meet requirements. At least one quarter of the bench-space can be devoted to general bacteriological examinations (including the routine throat-swabs). Another quarter can be laid out to deal with nothing but sputa (see page 221). A further quarter of the bench can be left clear except for a small gas-ring and an enamelled (or aluminium) saucepan for melting media and pouring plates, slopes, etc., of solid media as they may be required. This now leaves the remaining quarter of the bench for miscellaneous work and the accommodation of the incubator, hot-water baths, etc.

The General Bacteriology bench can be fitted as shown in Fig. 3, with a good-sized staining sink, eight 12-holed test-tube racks, a bunsen burner (with a pilot-flame), a rack for holding platinum loops and needles, and micro-slides. Let into the bench, 4 in. from the front edge, an oblong of plate glass 1 ft. 3 in. × 9 in., painted on its reverse side half black and half white, or sheets of ¼-in. "Vitreolite" of the same colours cemented in with bitumastic cement, may be fitted. The various

stains and reagents in routine use are set out on a shelf above the sink. The media-pouring bench should, if made of wood or lino-covered, be kept highly polished and frequently swabbed over with weak disinfectant. The shelf over this bench can be fitted with long narrow boxes divided into compartments to contain tubes of the various media in regular use, together with such reagents as may be required in this section.

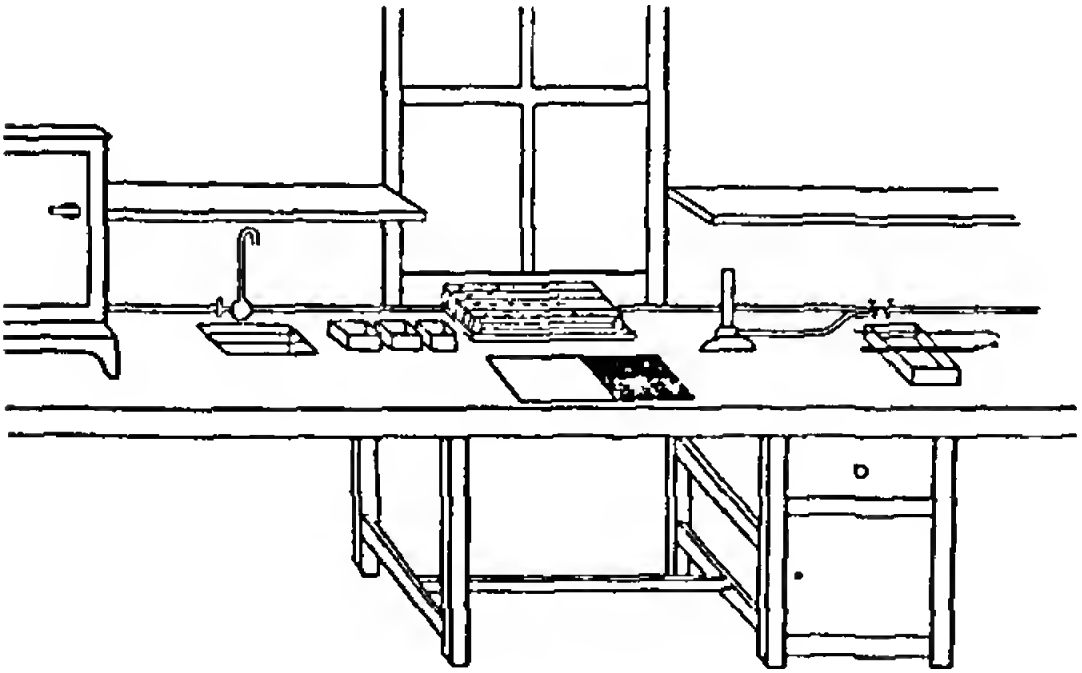


Fig 3
General bacteriological bench

A small drawer let in under the bench is useful for keeping notebooks and other odd personal effects. The cupboard should be quite shallow and possess well-fitting doors. The upper shelf of the cupboard should be used for storing poured plates, the next shelf, fitted with partitioned boxes similar to those over the media bench, for storing reserve quantities of tubed media. The lower shelves of the cupboard can be used for storing pieces of apparatus that are required only occasionally.

Considering how rarely direct daylight is used for microscope illumination, it is surprising to note the frequency with which this instrument is placed in front of a window! Far better is it to select the darkest corner of the bench and to instal the microscope there more or less as a permanent fixture. There

is less likelihood of it being disturbed to make room for other work; eyestrain is greatly reduced, particularly when long periods of time are spent at the microscope and, as one is taught to keep *both* eyes open when using the instrument, the attention is not diverted by other brightly illuminated objects near by. When the microscope is given a permanent position on the bench there is little point in wasting further space with elaborate lamps (which invariably get disturbed and need re-focusing). A small hole, 1 to 1½ in. in diameter can be drilled through the bench and an adjustable lamp installed beneath it. The mirror of the microscope may then be dispensed with and there are no extraneous beams of light to distract attention. A small piece of blue glass let into this hole, flush with the bench-top, will suffice to cut out the yellow bands from an ordinary electric light bulb for ordinary routine work, or colour filters fitted for special purposes.

Once the microscope is placed in position a mark is made on the bench round the base of the instrument for future positioning should it become disturbed. A bell-jar makes an excellent cover for the microscope. (For further notes on the microscope see chapter XXXI, page 264.)

There seems to be a wide diversity of opinion regarding the height of work benches. A low bench at which the worker is able to use an ordinary chair is, perhaps, more comfortable: but if this height is adopted it becomes necessary to sit down to every job and involves much stooping. A bench 3 ft. high enables the worker to stand or move along it whilst working without undue stooping and a stool approximately 2 ft. 3 in. in height provides reasonable comfort and permits of rising from it to move along the bench with greater ease. It is important to remember when installing mechanical apparatus such as centrifuges and shakers that even the smoothest running of these create a degree of vibration [illegible] take[illegible] parti[illegible] ...ope. The larger types of centrifuges are better bolted to the floor.

Frequent ablution is very much a part of the technician's daily routine and provision for this must not be overlooked.

A large sink (of the kitchen type) up to 3 ft. × 1 ft. 6 in. can serve the dual purpose of ablution and for washing apparatus which does not require to be sent to the cleaning room. If hot water is not "laid on" a large water-heater (Ascot type is recommended) should be installed over this sink. It is very important that each work room should have adequate ablution facilities; if workers have to use wash-basins in the usual premises providing them in other parts of the building, door-handles can become infected if hands are accidentally contaminated at the bench.

Premises allocated for laboratory accommodation vary enormously according to individual circumstances and it is therefore impossible to lay down any hard and fast rules as to its planning but the foregoing may serve as a general guide, and is adaptable.

Laboratory Benches

Modern plastic compounds are being utilized to-day for laboratory bench-tops and, no doubt, they have much to commend them. Nevertheless, few laboratories will replace existing benches, many of which have stood the test of time and are made from well-seasoned woods or are covered with good quality linoleum. Many laboratory workers, despite the modern trend towards plastics will continue to favour teak. This, the best of all materials for the purpose, will be met in many laboratories for years to come.

No doubt many benches have not received the care and attention due to them and are showing signs of wear and abuse.

Paints and varnishes should be avoided for treating bench-tops! However excellent their quality they cannot do more than provide a thin veneer on the surface which can be easily penetrated by either warm flasks or fragments of glass, leaving weak spots in the surface which can allow acids or alkalies, if spilt, to seep into the unprotected wood beneath.

The following are procedures for the treatment of bench-tops both old and new:

Old benches which have become badly marked and pitted should receive the attentions of a good carpenter or joiner

armed with a " sanding machine ". The entire surface of the bench should be removed down to bare wood and then be treated as for new benches (see below).

Old wood or lino-covered benches that have become discoloured and grimy from successive treatments with a variety of polishes over a long period should be treated with a liberal washing of benzene or xylol residues to loosen as much of the old polish as possible, followed by scrubbing with warm water and a good wetting agent such as one of the sulphonated fatty alcohols or covered with a solution of either (*a*) trisodium phosphate, 1 lb. in 1 gal of warm water, or (*b*) sodium metasilicate 1 lb. in 1 gal. of warm water, and allowed to stand for twenty to thirty minutes. They should then be rinsed off with copious warm water and allowed to dry. This treatment should be repeated if the surface is still greasy. From then on the benches may be treated as for new ones (see below).

New Wooden Benches

According to the colour required the wood may be stained with one of the following solutions, taking care to spread evenly and mopping off any surplus liquid with swabs of absorbent cotton wool.

Red Mahogany	Sudan red	7	grms.
	Pylakrome black (319)	10	"
	Azo orange (30)	4	"
	Dissolved in 1 pint of benzene or turpentine.		
Brown Mahogany	Azo oil yellow (408)	7	"
	Pylakrome oil green (430)	3·5	"
	Sudan red	3	"
	Azo orange	7	"
	Dissolved in 2 pints of benzene or turpentine.		
Walnut	Azo oil yellow	7	"
	Sudan red	0·5	"
	Azo orange	4	"
	Pylakrome green (430)	1	"
	Dissolved in 1 pint of benzene or turpentine.		
Oak	Azo yellow	15·5	"
	Pylakrome black (319)	0·5	"
	Dissolved in 2 pints of benzene or turpentine. (Repeat for increasing the depth of shades.)		

The woodwork should be allowed to dry thoroughly and then, with a large paint brush a solution of one part linseed oil to two parts turpentine may be applied. Excess should be avoided; two or three applications over a period of time will soak well into the wood, whilst a lavish coat tends to form a skin which will lie on the surface. The wood should be allowed to dry for at least forty-eight hours and then, using a soft *wet* cloth small quantities of one of the polishes referred to on page 12 may be rubbed well into the surface and allowed to stand for twenty-four hours. An attempt to obtain a high gloss-finish should not be made until at least three applications, over a period of at least seventy-two hours, have been made. After this the woodwork may be polished with a soft dry duster using short circular movements of the hand over the surface, a small area at a time. A light application of the same polish once a fortnight should maintain the bench tops in good waterproof condition.

New Linoleum-covered Benches

Using a soft clean cloth, an emulsion of linseed oil and oleic acid should be rubbed well into the lino and allowed to dry thoroughly. This should be repeated after forty-eight hours, afterwards treating with the lino-polish referred to on page 12.

Draining Boards and Racks

Old boards and racks should be scraped and sandpapered down to bare wood and then treated as for new ones (see below).

New wooden draining boards and racks should be rendered impervious to acids and alkalies and also be made waterproof by applying two or three coats of the following solution at intervals of twenty-four hours:

Copper sulphate	4 per cent.
Ferrous sulphate	2 „
Potassium permanganate	8 „
Water to dissolve.	

The solution should be applied hot.

When dry the surface should be rubbed briskly with a rough cloth, after which two coats, again at twenty-four-hour intervals, of

Aniline	12 per cent.
Hydrochloric acid	18 „
In water	

are applied. The woodwork should then be thoroughly washed with warm water, rubbed down with a rough cloth and allowed to dry. Two coats of linseed oil, thinned with a little turpentine, should be rubbed well into the grain of the wood and again allowed to dry. This treatment leaves the wood a matt black. It should be treated with linseed oil thinned with turpentine about once a month to maintain its waterproof quality. No polish or other preparations should be used.

Polishes

For wood:

(1) Carnauba wax	1 lb.
Beeswax	½ lb.
Ceresin	½ lb.
Turpentine	½ lb.
Naphtha	½ lb.
Stearic acid	½ lb.
Triethanolamine	2 oz.
Water	2 lb.

The waxes are melted and the stearic acid added at 90° C. The naphtha is then added slowly. The water is heated to boiling point and added very slowly to the mixture, stirring vigorously all the time until an even emulsion is obtained. The stirring is continued slowly until the polish is cold. A mechanical stirrer is strongly advised for this purpose. The polish requires hard rubbing but produces a high lustre.

(2) Cobblers' wax (heelball) is procurable almost everywhere at a few pence per stick. Four sticks of this are melted by gently heating on a water bath and 1 pint of turpentine added, stirring the while. The lighter shades of brown should be chosen when purchasing the heelball to avoid deepening the colour of the woodwork. The polish is applied thinly, with a damp cloth, allowing it to dry thoroughly before polishing with a soft dry duster.

For linoleum:

Carnauba wax	1 lb.
Paraffin wax (m.p. 60° C.)	1 oz
Ceresin wax	7 oz.
Turpentine	1 pt

The waxes are melted together by [illegible] added slowly, stirring [illegible] a damp [illegible] to c[illegible]

CHAPTER II

CARE OF LABORATORY APPARATUS AND EQUIPMENT

Cleaning Glassware

Most of us, at some time or other, have taken a share in the commonest of all household chores—washing up! The washing and cleaning of laboratory glassware, however, must not be looked upon as just a glorified form of this most uninteresting task. From start to finish, from the arrival of new apparatus to its, perhaps inglorious, end at the hands of some inexperienced person, laboratory apparatus should be treated as something rather sacred. It is not enough to subject it to similar treatment as is meted out to the household china in the kitchen sink, finished off with a vigorous polish with a not-too-clean cloth.

It is a wise plan to make the sterilization of all laboratory glassware a matter for routine procedure; that is, irrespective of whether apparatus has been used at the bench or just arrived from stock, it should be made a hard and fast rule to immerse it in a weak disinfectant solution such as 5 per cent. lysol (which has the advantage of a soap base to assist in removing grease) or one of the modern soap substitutes, or 2 per cent. solution of sodium hypochlorite (eclipse) with a small amount of lysol
[illegible] utes and then cleaning in the
[illegible] adhering rigidly to such a practice any possibility of overlooking infected apparatus is eliminated.

All laboratory glassware, before it can be brought into use, must be free from all traces of chemical and organic contamination and to ensure this special methods for cleaning it have been devised. To short-circuit the process of cleaning is but to invite disaster at some stage or other of subsequent work performed with it. For primary sterilization of infected glassware the autoclave is to be preferred as any traces of organic matter that may possibly have been overlooked would

be "baked" on to the glass if it were subjected to dry heat.

Flasks, Beakers, Large Bottles, etc.

These types of glassware can be cleaned by rinsing well under running water to remove as much of the contaminating material as possible. They are then washed in warm soapy water, using a stiff bottle-brush (an assortment of which should be kept hanging by the sink). They are again rinsed in running water, filled to the rim with weak potassium permanganate solution and allowed to stand overnight. The permanganate solution is then poured into the bowl labelled such (see Cleaning room, page 2), the vessel again rinsed and a small quantity of strong hydrochloric acid poured in. The acid is allowed to run over the entire inner surface of the vessel by tilting and rotating it slowly. The acid is then poured away and the vessel allowed to stand for a few minutes to allow the film of acid to complete its work of removing any adhering particles of oxidized material left by the permanganate solution. Again the vessel is rinsed in running water and then placed on the draining rack to dry.

Petri-dishes

It should always be made a routine practice to separate petri-dishes and lids at the work-bench before immersing them in the lysol bucket or other container for discarded cultures. When the dishes arrive at the cleaning room they must first be autoclaved with the remainder of the infected material from the laboratory and then thoroughly washed in warm soapy water followed by rinsing in running water. They may then be stacked along the draining board. When almost dry they should be dipped separately into a bowl containing methylated spirit and again stacked to dry on large sheets of filter paper on the bench: after which they should be polished with a clean grease-free glasscloth. The lids and lower halves are then matched up ready for sterilizing. It is most important that petri-dishes, in fact *all* glassware, should be thoroughly dried before consigning them to the hot-air sterilizer, as being of

comparatively uneven glass, any dampness will cause heavy casualties amongst them.

Test-tubes

Although there is an almost infinite variety of test-tubes in use in most laboratories, the process of cleaning them is more or less the same. The exception to this being for tubes which are used for the Wassermann, Kahn and similar serological work. Upon removal from the autoclave the tubes should be carefully placed in an enamelled bowl containing hot soapy water, a few at a time to avoid breakages. There is no really efficient way in which to clean test-tubes in bulk and each tube should be dealt with separately. According to its size, a test-tube brush should be inserted whilst the tube is immersed, and the inside scrubbed briskly. The soapy water should be drained out and the tube rapidly rinsed under the tap and inspected for any remaining traces of contamination. The scrubbing may be repeated if necessary. The tubes should then be plunged into another bowl standing under the running tap. Rinsed tubes may be stacked, open end downwards, in wire baskets and allowed to drain. When quite dry they are removed to the bench for plugging and sterilizing or to be fitted up again into any special apparatus of which they may form part. Should test-tubes be required urgently, after washing and rinsing they may be plunged into a bowl of methylated spirit and drained.

Screw-cap bottles

These may be treated as for test-tubes. The screw-caps are a different problem however! Each screw-cap contains a "liner" of some description or other and except where these are of rubber, all liners should be discarded and new ones used each time the bottles are cleaned and prepared for re-use. The metal caps should be washed in warm soapy water, rinsed thoroughly and allowed to dry rapidly to avoid corrosion. The bottles and caps are then removed to the bench for re-fitting and sterilizing. If rubber liners are used, these may be washed, rinsed, dipped in methylated spirit and laid out to dry; after

which they may be re-inserted into the caps. NOTE.—Sterilization must be done in the autoclave when rubber liners are used.

Pipettes

Two tall wide-mouthed jars (cylinders for preference), one filled with a weak solution of potassium permanganate and the other with strong hydrochloric acid may be used for cleaning pipettes of both the graduated and bulbed types. The soiled pipettes are first rinsed under running water, making sure that a good jet of water passes through them to remove the bulk of the contaminating material, then placed, point uppermost, in the potassium permanganate solution for two or three hours (overnight is more satisfactory). On removal from this solution they are again rinsed in running water and placed in the hydrochloric acid (taking care to see that no air-bubbles are trapped in the pipettes) for one hour. The acid is drained off into another jar and a rubber tube attached to the tap inserted to the bottom of the jar containing the pipettes and a stream of water allowed to run up through the pipettes. When the bulk of the acid has been washed away it is safe to lift the pipettes out, a few at a time, and a jet of water directed through each pipette separately. With an ordinary washbottle a small quantity of methylated spirit is next run through each pipette, allowing the spirit to run into a vessel placed under the pipettes (this spirit can be re-used). The pipettes are then placed in the racks to drain dry.

Specimen bottles

Small bottles used for the collection of specimens such as sputa, stools, etc., may be treated similarly to test-tubes, except that those with "shoulders" should be stood, mouth downwards, on large sheets of filter-paper to drain.

Wassermann, Widal, Kahn and other tubes used for serological work

Stout rubber bands (rings cut from an old cycle inner-tube serve the purpose) may be used to bundle these tubes together for cleaning.

Method A. Bundles of tubes (with the open ends all facing in one direction) are rinsed in running water and, using a rubber glove kept for the purpose, immersed, open ends uppermost, in the potassium permanganate bowl where they are allowed to remain overnight. The bundles are next removed, the permanganate solution allowed to drain out, and the tubes rinsed under running water; after which they are placed, again open end uppermost, in the hydrochloric acid bowl where they remain for one hour. They are then removed, the acid drained back into the bowl and the tubes rinsed thoroughly under running water. The water is drained out for a few minutes and the bundles of tubes plunged into the bowl containing methylated spirit. The spirit is drained back into its bowl as each bundle is removed and the bundles stood, open end downwards on sheets of clean filter-paper to drain dry. All tubes, particularly those used for flocculation tests, must be carefully scrutinized before being put back into use The interior of the tubes should be polished with small wads of cotton wool or fine tissue paper attached to wire holders.

Method B. The bundles of tubes are immersed in a 2 per cent. solution of sodium hyperphosphite (calgon) in an enamelled bucket and allowed to boil gently over a gas-ring for thirty minutes. The bucket is then transferred to the sink and a length of rubber tubing, attached to the tap, is inserted to the bottom of the bucket and the water turned on (slowly at first) and allowed to run until the calgon solution is washed away. The bundles of tubes are then drained, immersed in a 1 per cent. solution of hydrochloric acid, rinsed thoroughly under running water and placed in the methylated spirit bowl. They are then drained on sheets of filter-paper followed by inspection and polishing as before.

Corks

Except for those used for "T.B." sputum bottles (which should invariably be burned) corks may be used a number of times before being discarded. Those recovered from the lysol-buckets should be washed very thoroughly in warm soapy water, tipped into a galvanized wire basket standing in a bowl

under a running tap and allowed to rinse for at least half an hour. They are then spread out to dry. Rubber bungs and screw-cap liners may be cleaned in a similar manner, remembering that rubber must not be subjected to any great degree of heat when drying.

Microscope slides

As final decisions in diagnosis are frequently made upon what is seen on a micro-slide under the microscope the necessity for scrupulous cleanliness cannot be overstressed. Fortunate, indeed, is the laboratory which can afford to use a micro-slide once only and then "scrap" it. Much has been written regarding the cleaning of microscope slides; two methods only are given here.

Method 1. Slides are immersed in 10 per cent. potassium dichromate in 10 per cent. sulphuric acid in a heavy-enamelled iron bowl and boiled for twenty minutes. They are then flooded with running water (without pouring off the dichromate solution) to "float off" the scum which forms on the top of the fluid; allowing the water to run until all traces of the dichromate have been removed. The slides should be stirred gently from time to time during the process to ensure that traces of dichromate do not remain trapped between slides. The slides are next lifted out, a few at a time and spread out to dry. They are then polished with a clean grease-free glass-cloth. The dichromate solution gives off acrid fumes which are a discomfort to the worker, and a fume-cupboard should be used for the boiling process. A certain amount of bumping occurs during boiling which is apt to leave scratches and blemishes on the surfaces of the slides.

Method 2. All slides that have had oil or canada balsam on them are first dropped carefully into the jar containing xylol and left for at least thirty minutes according to the length of time that the oil or balsam has been left on (see page 3). The xylol is poured into an empty jar and methylated spirit poured on to the slides. The slides are stirred gently to ensure the spirit penetrates between them, and allowed to stand for a

further thirty minutes The spirit is then poured off into the jar that is now empty and the jar containing the slides placed under running water, stirring them occasionally. NOTE — Some " milkiness " on the surface of the slides can be ignored at this stage. The slides are next removed, a handful at a time, allowed to slide separately into a bowl containing a 2 per cent. solution of sodium hyperphosphite (calgon) and allowed to simmer gently over a low flame for thirty minutes. The bowl and its contents are next transferred to the sink and a slow jet of water, through a rubber tube reaching to the bottom of the bowl, is allowed to run until all traces of the calgon are removed. The water is poured off and 1 per cent. hydrochloric acid added. The slides are next lifted, a few at a time, from the bowl, dipped in methylated spirit and polished on a clean grease-free glasscloth. To ensure that no traces of grease remain, the slides may be stacked loosely on the asbestos shelf of the small oven and baked at 180° to 200° C. for thirty minutes.

Cover-slips

These delicate little " wafers of trouble " are a source of annoyance to those responsible for cleaning them, and a constant anxiety to whoever has the task of maintaining stocks. Nevertheless, many which would otherwise be destroyed can be recovered if a little forethought is given to the subject of their recovery and cleaning. Wherever possible cover-slips should be removed immediately specimens are finished with and each work-bench in the laboratory should be equipped with a small lidded pot into which they can be dropped These pots should be sent to the cleaning-room periodically and their contents treated in a manner somewhat similar to that for slides up to the final dip in methylated spirit; after which they are stored in clean spirit until required for use. When required they are removed from the spirit, polished on a soft glasscloth (or if available, a silk handkerchief) and, held in forceps, passed rapidly through the bunsen flame to remove all traces of grease. (NOTE —The latter must be done very rapidly to avoid warping the glass.)

Throat and similar types of swabs

Immediately swabs are finished with at the work bench, the infected swabs are removed from the tubes and dropped into the lysol-bucket where they float swab downwards. On arrival at the cleaning-room they are autoclaved with the remainder of the infected materials and it then becomes an easy matter to remove the cotton-wool from the ends of the wires by "pinching" a piece of lint on to the wire and drawing the wire through it. The lysol-soaked wool comes away cleanly. The wires, complete with corks are stacked in wire-baskets, rinsed thoroughly under running water and allowed to dry. They are then transferred to the bench for re-making (see page 30).

Boring Corks

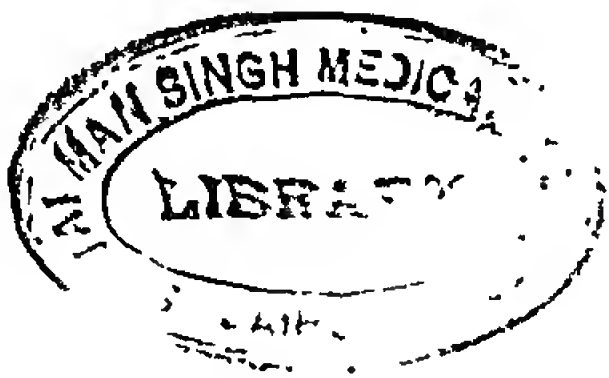
For both rubber and ordinary corks boring should always be commenced at the narrow (tail) end of the cork. Having started to bore, the borer is withdrawn slightly and a small quantity of (*a*) methylated spirit for ordinary corks, or (*b*) glycerol for rubber corks, poured into the borer. Boring is then resumed, using a twisting motion of the wrist and with the cork pressed firmly against a *perpendicular* flat soft wood surface. Great pressure should not be exerted on the borer which should be given opportunity to *cut* through the cork rather than *punch* the hole. The twisting motion should be kept constant and, at intervals, the borer should be withdrawn slightly to allow fresh spirit or glycerol to seep under the cutting edge. Too much pressure on the borer will result in cone-shaped holes, or, with ordinary corks, fractured surfaces inside the hole. The perpendicular surface chosen should be one of no import as the cork should be kept pressed to it until the borer has passed right through the cork into the wood of the surface, ensuring clean sharp edges to the hole.

Labelling Reagent Bottles

Few technicians include signwriting amongst their accomplishments and many shelves contain a miscellany of oddly-labelled (some almost illegible) bottles. A small stencil ("UNO", procurable at most stationers) with ½-in. letters and

figures, together with the special pen and a bottle of indian ink are sound investments.

Many methods for preserving reagent bottle labels have been devised most of which involve a lengthy process of impregnating the labels with either agar or gelatin, drying and varnishing with paper-varnish. Transparent "DUREX" cellulose tape, obtainable in various widths, is a definite improvement upon the older methods. Strips of the tape are cut, a little wider than the labels, and pressed firmly over them. "Dispensers", for controlling and cutting the tape as desired, are also available at a reasonable price.



CHAPTER III

PREPARING ROUTINE GLASSWARE AND APPARATUS FOR STERILIZATION

A SOMEWHAT libellous joke is current in laboratories regarding the popularity of dark-coloured bottles for use in storing vaccines! It is said that the bottles prevent patients from seeing the straw and other odds and ends left in the bottles. Be that as it may, it is an indisputable fact that of all preparation work in a Bacteriological laboratory, that of sterilizing is one of the most important. All traces of contamination which may evade the drastic cleaning processes, together with microbes which are constantly dropping into everything in use are "put paid to" by efficient sterilization after vessels are sealed, corked, plugged or capped. When dealing with laboratory apparatus alone we are able to destroy these intruders by subjecting the apparatus to high temperatures for prolonged periods of time. When, however, dealing with substances which are, themselves, destroyed by excessive heat, other methods must be employed and these are dealt with separately in the chapters on Culture Media and its Preparation and on Special Apparatus.

Any apparatus of which rubber forms part must always be sterilized by moist heat; most other laboratory glassware may safely be consigned to the hot-air oven, a simple device rather like an ordinary cooking oven, but thermostatically controlled and provided with a thermometer. 150° to 160° C. for 1½ hours from the time that the thermometer registers these temperatures is sufficient.

When preparing apparatus that requires labelling subsequently, such as sputum-pots, throat-swabs, etc., the labelling should always be done after sterilization. Even so, when, later, some of the apparatus is destined to spend a while in the incubator, the ordinary plain white labels tend to peel off when the glassware contracts upon removal from the incubator. Such pieces of apparatus are best labelled with strips of gummed

paper which will go completely round the vessels and just overlap (gummed paper tape of the kind which is almost universally used for packing is admirably suited for the purpose). The tape is cut into lengths according to the circumference of the vessels. Clear cellulose paints and paper-varnishes are sometimes recommended for painting over labels to prevent them peeling but these have the disadvantage in this instance of being very difficult to write on.

Test-tubes

Tubes are plugged with cotton-wool (non-absorbent) so that the wool does not penetrate into the tube for more than 1 in., nor is there an excess of cotton-wool loose above the rim of the tube, the wool above the rim should be just sufficient to grasp with the little finger when removing it whilst the tube is in use. The tubes are then packed loosely into wire baskets, placed in the hot-air oven so that the cotton wool does not touch the sides of the oven at any point.

Petri-dishes

Special carriers for these are an advantage and are obtainable from most laboratory furnishers. These consist of tall copper cylinders fitted with sliding racks and well-fitting lids. The dishes are loosely packed into the racks which are then slid gently into the containers and the lids replaced. The containers are stacked in the hot-air oven. Upon removal from the oven a strip of gummed paper bearing the date of sterilization is pasted round both the lid and container. It should be stuck so that it is necessary to break it when the container is opened.

Pipettes

Graduated and bulbed pipettes that are to be sterilized are wrapped separately in thin " kraft " paper. For this narrow strips of the paper are cut, measuring approximately 18 in. to 2 ft. long by 1½ in. wide. Commencing at the delivery end (or point) of each pipette, the strip of paper is wound round the pipette in a tight spiral, each turn overlapping the previous one,

leaving a loose end which is folded over the mouthpiece (or "open" end) of the pipette and secured either with a strip of gummed tape, or, better, with an elastic band.[1]

The wrapped pipettes are bundled together loosely in an old petri-dish carrier (or specially tall copper cylinder made for the purpose) and placed in the hot-air oven.

Specimen bottles

Well-fitting corks are placed very loosely into the necks of the bottles which are stood upright on enamelled trays and placed in the hot-air oven. It is important that the corks should be quite loose whilst the bottles are in the oven otherwise when heating, there will be a series of minor explosions during sterilization caused by the air in the bottles expanding and blowing the corks out. Immediately the oven is cooled sufficiently, the bottles are removed and the corks pressed well into the necks.

Faeces-pots

These may be treated as for ordinary specimen bottles (see Special Apparatus, page 31).

Throat-swabs

These are treated as for specimen bottles, except that they may be loosely packed in wire baskets whilst being sterilized.

[1] [illegible] should end at the mouthpiece [illegible] use, this end may be held in [illegible] wrapping.

CHAPTER IV

SPECIAL APPARATUS FOR THE COLLECTION OF SPECIMENS

It is not always possible for the patient to come to the laboratory neither is it always practicable for the pathologist or his assistant to go to the patient! It therefore rests with the patient's medical adviser to collect the specimens he requires to be examined and to forward these, together with his observations to the laboratory Similar conditions prevail with regard to collection and submission of food samples, etc. As this frequently entails use of the postal services, it is in the interests of the laboratory, in fact, of all concerned, for the laboratory to take the responsibility for providing suitable containers and materials to ensure the safe transport and good condition of specimens upon their arrival. Again, pathological specimens seldom, if ever, improve with keep, and it therefore becomes necessary to make provision for the preservation of certain specimens during transit. There is little point in dwelling at length upon the subject of outer covers and wrappings for specimens as most firms of repute dealing with laboratory requirements supply a wide range of these and it becomes a matter of individual choice by those concerned as to which are used. It must be borne in mind that definite regulations regarding the transit of pathological materials are laid down by the postal authorities. We will restrict ourselves here to the actual outfits that can be produced at the laboratory and supplied to doctors on request. The body-fluids present the greatest problems and of these, blood is perhaps the greatest difficulty of all as the tests to which this may be subjected are of almost infinite variety. For some tests blood must remain unclotted, whilst for others it must be allowed to clot and for yet others it must be introduced into preparations that will render its bactericidal powers ineffective and so on!

Blood-count outfits

Whilst blood-dilution pipettes may be of great use in the laboratory, the risks of leakage and inaccurate filling render these unsuitable for issue. Thick-walled tubes measuring $3\frac{1}{2} \times \frac{3}{8}$ in. are set out in racks and into each is placed 1·0 ml. of distilled water. With a writing diamond a small mark is made on the side of each tube at the bottom of the miniscus of the water and the tube inverted to drain dry. Into each tube is measured exactly 0·1 ml. of 10 per cent. sodium citrate solution and a small rubber bung fitted to the tube. The tubes are next packed into wire baskets and autoclaved at one atmosphere for thirty minutes. (NOTE.—When autoclaving any apparatus which is tightly corked or actually sealed it is absolutely essential to allow the autoclave to cool thoroughly before it is opened otherwise there is grave danger of the vessels exploding owing to the fluid within them boiling under reduced pressure.)

On removal from the autoclave the tubes are labelled, with the lower edge of each label touching the small mark on the side of the tube. The labels should be printed (written or marked with a rubber stamp): "BLOOD UP TO LOWER EDGE OF LABEL ONLY; NO MORE", leaving sufficient space clear on the label to add the patient's name, etc.

Blood-culture outfits

Method 1. The outfit consists of two tubes: "A"—designed to allow the medical practitioner to introduce blood directly into the fluid medium in which the blood is to be "grown"[1] and "B"—to submit to the laboratory a tube of unclotted whole blood. Tube "A" measures 6 in. × $\frac{5}{8}$ in. and tube "B" 5 in. × $\frac{5}{8}$ in. Into tube "A" is placed 10 ml. of nutrient glucose broth, and into tube "B" 0·2 ml. of sodium citrate (10 per cent. solution). All tubes are plugged with cotton-wool and fractionally sterilized. A number of rubber vaccine caps are placed in a clean enamelled saucepan, covered with distilled water and allowed to boil vigorously for thirty minutes after

[1] A loose laboratory expression meaning to incubate to encourage the multiplication of any microbes present

which they are allowed to continue simmering over a low flame whilst the tubes are placed out in test-tube racks and the cotton-wool plugs burned by passing a bunsen flame along them. One at a time the vaccine caps are removed from the saucepan with sterile forceps (crucible tongs are useful for this) and transferred to the other hand, holding the outside of the bulbous part of the cap between the thumb and finger (avoiding touching the rim of the cap). With the same forceps, the cotton-wool plug is removed from one of the tubes and the rubber cap fitted over the mouth of the tube and pressed firmly on until the rim of the tube can be felt touching the top of the cap. All tubes, both " A " and " B " are dealt with in this manner.

Short lengths of fine copper wire are cut and one length is twisted round the cap on each tube just above the thick rim of the cap, and tightened with small pliers until the rubber is held firmly to the tube without being cut into by the wire. With the aid of a sterile 5 ml. or 10 ml. hypodermic syringe fitted with a fine needle 0·5 ml. of " Injectio Trypsin " may be added to each " A " tube by first flaming the top of the rubber cap, piercing it with the needle and squirting the trypsin into the broth. Whilst the volume of trypsin added is really negligible in this case, positive pressure inside the tube can be avoided by removing an equal volume of air whilst the needle is still inserted.

Method 2. With the recent introduction of small screw-cap bottles fitted with caps with a small hole pierced in the top, and with rubber liners, the above technique may be modified. The bottles, of approximately 28 ml. capacity, are sterilized fractionally with the screw-caps loosely fitted. At the completion of the sterilization the caps are screwed tight and the " Injectio Trypsin " introduced into the " A " bottles through the rubber liner with a syringe as before.

Exudate outfits

These are used mainly for venereal work. Success with the preparation of these outfits depends largely upon the skill at glassblowing of the person preparing the capillary tubing used. Laboratory glassblowing can only be

learned by practical experience and, except for a few hints interspersed throughout these pages, it must be left for the reader to acquire skill in this direction under the guidance of skilled colleagues. Capillary tubing of approximately 1·0 mm. bore is drawn out from oddments of glass tubing and cut into 3-in. lengths. Two or three of these lengths are placed into each of a number of small test-tubes ($3\frac{1}{2} \times \frac{3}{8}$ in.). The tubes are plugged with cotton-wool and sterilized in the hot-air oven. When appropriately labelled the tubes are ready for use.

Microscope slides

It is sometimes necessary for microscope slides to be available for doctors to make slide preparations at a patient's bedside or at his surgery. A difficulty that frequently arises with such preparations is that, the specimen having been taken, the slides are placed together inside their container before the films are dry and, in consequence, by the time these arrive at the laboratory they are decomposed and useless. To avoid this, and to eliminate any elaborate packing, small thick gummed labels measuring 1 in. square are doubled over each end of each slide. Two slides are placed in each of a number of slide boxes (these boxes, measuring $3\frac{1}{4} \times 1\frac{1}{4}$ in., may be obtained from most laboratory furnishers). It will be noted that the thickness of the labels is just sufficient to prevent films from touching anything else, whichever way the slides are placed in the boxes.

Tubes for collection of clotted blood

Small screw-cap bottles are frequently used for this purpose, but thick-walled *rimless* tubes, 5 in. × $\frac{5}{8}$ in., with well-fitting corks or rubber bungs are admirably suited; particularly as square-sectioned boxes, with cylindrical interiors to fit the tubes are obtainable quite cheaply. Numbers of these boxes can be packed neatly in the minimum space for transport. As in the case of the bottles, tubes should be lightly corked if using the hot-air oven for sterilization, but, if using rubber, they may be tightly corked and steam-sterilized. Upon removal from the sterilizer, the tubes are labelled and boxed ready for use.

Wright's blood-capsules

Here, as for exudate tubes, the skill of the worker in preparing the apparatus is a deciding factor in the successful collection of blood. Glass tubing of 4 or 5 mm. bore is drawn out at 5-in intervals along its length as shown in Fig. 4, and cut into lengths leaving a short nozzle at each end of each length. The centre of each of these lengths is next softened in the blowpipe flame, drawn to a similar capillary

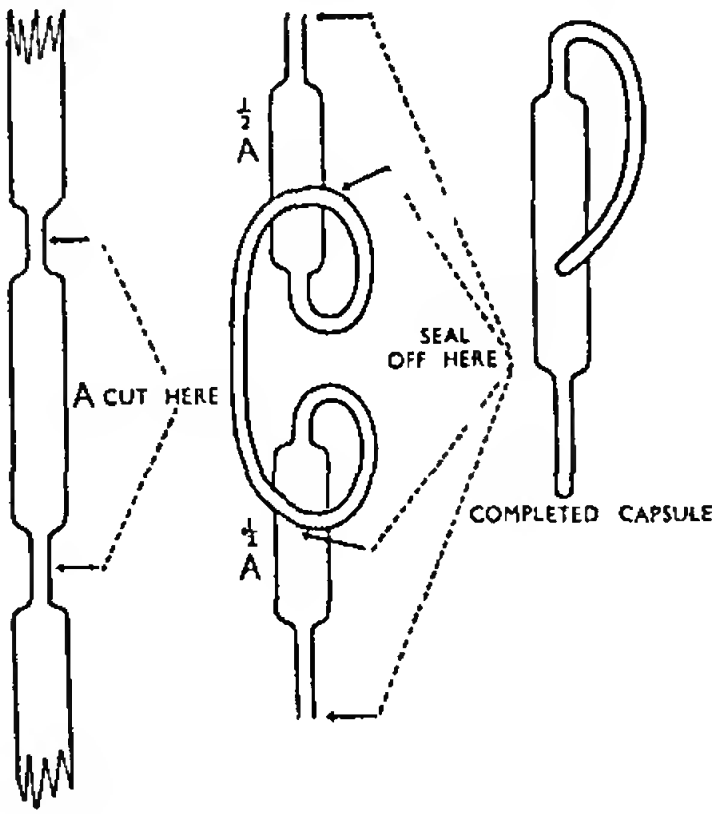


Fig 4
To make Wright's Blood Capsules

and, by a deft twist of the wrists, the capillary thus made is doubled back on itself and slightly curved out of horizontal as shown in the diagram The curved capillary is melted through in a fine fierce flame, sealing the two ends as the capillary is severed. The straight capillaries at the other ends of the two capsules are also sealed off in the flame. The capsules, after precautionary sterilization in the hot-air oven, are now ready for use. Should the capsules be required for collection of unclotted blood, a few drops of 10 per cent. solution of sodium citrate may be introduced with the aid of a fine capillary

pipette inserted into the straight end just prior to sealing it. Great care must be taken to avoid wetting the sides of the opening in the process as this makes it difficult to seal the capsule. For capsules containing sodium citrate solution it is necessary, of course, to sterilize in the autoclave. (See Laboratory Glassblowing, page 32.)

"Oxalate" tubes

An expression frequently used in the laboratory is a "knifepoint". It is a very loose term to describe the approximate quantity of a substance to be used when an exact amount is immaterial. A "knifepoint" of small crystals or powder is the amount that can be picked up on the end or point of a small scalpel (pocket-knife or nail-file) to a distance of about ¼ in. along the blade.

To ordinary blood-collection tubes a "knifepoint" of potassium oxalate crystals is added. The tubes are then tightly corked (rubber-bungs are preferable) and autoclaved. Labels marked "OXALATE ADDED—Blood up to lower edge of label ONLY" are fixed to the tubes so that the lower edge of the label indicates approximately the 5 ml. level in each tube.

Throat-swabs

In normal times, aluminium wire of $\frac{1}{16}$-in. gauge is plentiful and can be purchased in large coils. Lengths of this wire are cut measuring about ½ in. longer than the tubes which are to be used. One end of each length is roughened by rolling it between two coarse rasps. One rasp is fixed to a board and the other fitted with a wooden handle. Two or three lengths of wire are held lightly on the fixed rasp and the other is drawn across them causing them to roll between the teeth of both rasps. Suitably-sized corks are bored with one hole with an ordinary bradawl and the unroughened end of a length of wire pushed through until it protrudes about 1 in. above the top of the cork (the wide end). With a pair of fine round-nosed pliers this end is bent double and pushed back into the cork until the bend fits flush with the top of the cork. This part of the swab should last for a considerable while and

merely need fresh cotton-wool and re-sterilizing. A common error when preparing swabs is the use of too much cotton-wool, making an untidy lump that either prevents the user from reaching where he wishes to get or else becoming unravelled in the patient's mouth, to his discomfort and the doctor's distress and annoyance. To prepare a serviceable swab a small wisp of cotton-wool is taken between the thumb and finger, the roughened end of a wire placed on the centre of this wisp and slowly twisted until the wool just, and only just, covers the wire. The cotton-wool is then bent over the tip of the wire and the twisting continued, allowing the wool to slip between the finger and thumb, but increasing the pressure gradually for a few moments. The swabs are then inserted into their tubes and sterilized in the hot-air oven. With a little practice well-made swabs can be rapidly produced which will reflect credit upon the laboratory and be a source of pride to yourself, to the doctor who uses them, and to your colleagues when the swabs are returned for examination.

Sputum-pots

Bottles or pots used for the collection of sputum should never be of the screw-cap type. The best container is the ordinary "specimen tube" measuring 3 in. × 1 in., and with straight sides and flat bottom Corks used for these should never be re-used, but, after thorough autoclaving, discarded (or safer still, consigned to the furnace). The tubes are loosely corked and sterilized in the hot-air oven, the corks being pressed firmly into the necks of the tubes immediately they are removed from the sterilizer. Small, square-section boxes with cylindrical interiors which loosely fit the tubes should be used for transmission of the tubes through the post. Sufficient of these boxes should be available at all times and any less secure form of transport avoided.

Faeces pots

Tubes similar to those used for sputum are best for the purpose of transporting faeces specimens. Small metal spoons are fixed into the inside end of the corks prior to sterilizing.

of approximately 2 mm. diameter and curved slightly as in the diagram. This capillary is sealed at its narrowest point. A short length of 3-mm. tubing is then cut and sealed at one end. With the blowpipe flame reduced to a fine point and with strong air pressure, a small area of the first tube is heated (at the wide part just below the convex side of the curve) until it is quite soft. A bubble of glass is blown at this point. Next

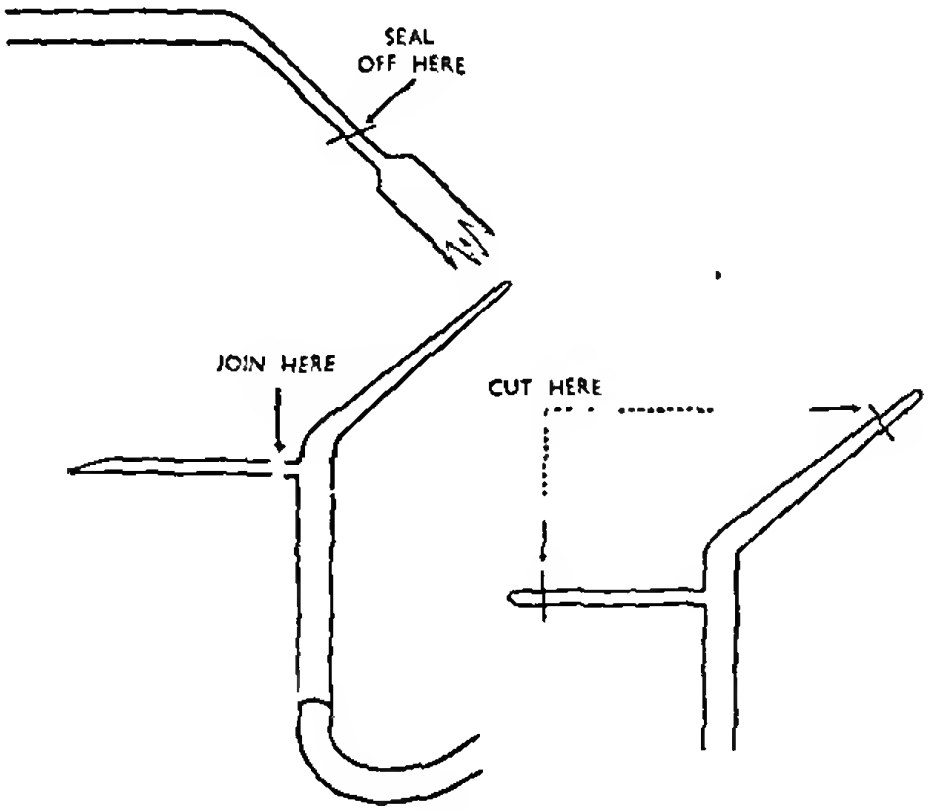


FIG 5

the bubble is broken, the tube returned to the flame and the edge of the hole softened until it is of even thickness. The blowpipe flame is then increased slightly and, holding this tube in one hand, and the small sealed tube in the other, both the hole and the open end of the small tube are softened. They are withdrawn from the flame and fitted together so that they form a "T". The blowpipe flame is adjusted to a fine point and, taking a small area of the join at a time, the glass is softened until the joint at each spot heated becomes completely fused together. At each stage gently blow and draw

at the open end of the first tube, watching the softened part of the joint all the time to ensure that too much pressure is not exerted This operation should be repeated until the whole joint is evenly fused. The joint is coated with carbon in the " white " flame of the blowpipe and stood aside to cool. The open end of the dropper is fitted through a suitably-sized bored cork and the sealed ends cut off to form the complete dropper shown in the diagram (Fig. 6).

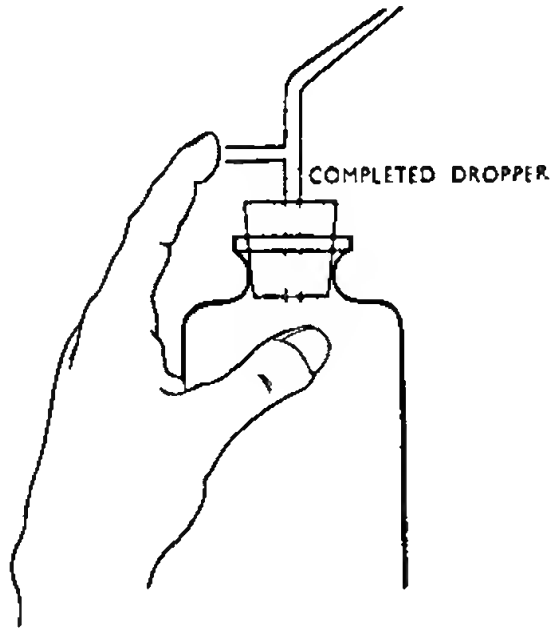


Fig 6

T and Y pieces

The technique for the dropper above may be slightly modified for making T and Y pieces. If making the pieces with wide-bore tubing, small corks may be introduced into the ends instead of sealing them during manipulation of the work.

To make Standard " Throttled " Micro-pipettes

A set of standard throttled micro-pipettes, for measuring out quantities of mercury with which to calibrate capillary pipettes, is a very useful addition to the equipment of any laboratory and can be easily made from oddments of 6 to 7 mm. diameter glass tubing. Short lengths of tubing are drawn out

it completely useless. As it does not come into direct contact with any fluids to be measured no need exists for washing it.

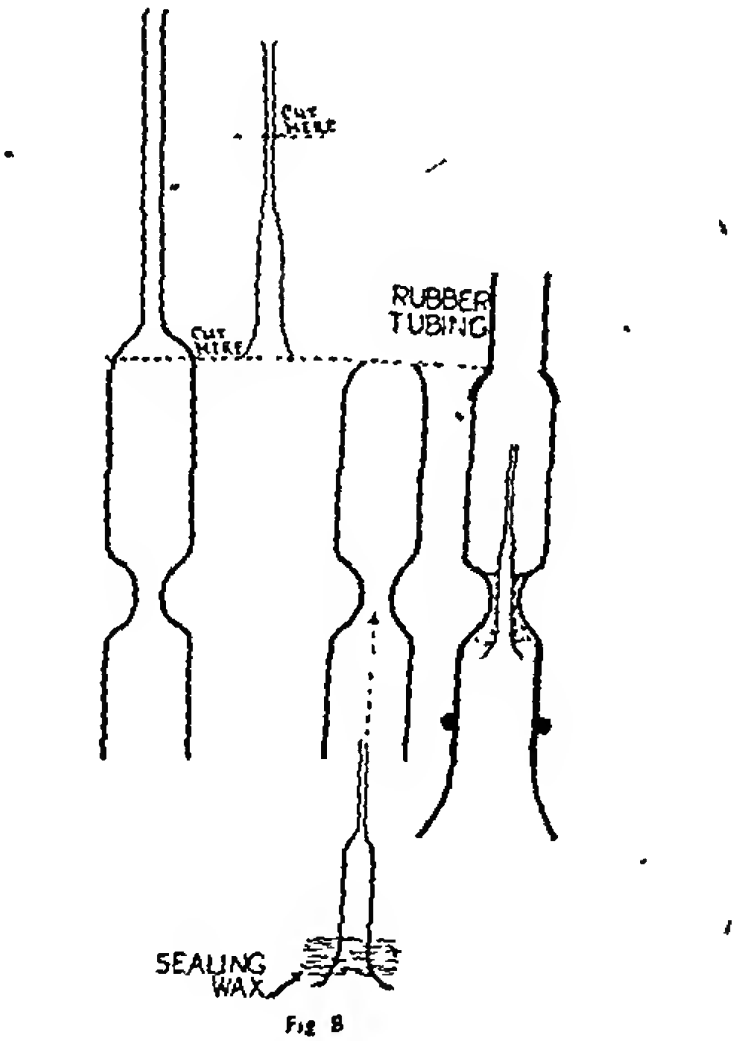


Fig 8

Cleaning mercury

Mercury rapidly becomes dirty owing to oxidization and as it is essential for it to be perfectly clean for calibrating pipettes it should be treated, just prior to use, as follows:

A small quantity is shaken in dilute nitric acid (10 per cent.) and then washed in several washings of distilled water.

To remove the water a filter paper is folded in the usual way and a small pin-hole pierced in the apex of the cone before inserting the paper into a clean, grease-free funnel. The wet mercury is poured into the funnel where the paper absorbs the moisture as the mercury passes through the pin-hole whilst

traces of dross will float on the surface of the mercury and be left behind in the filter. This process should be repeated several times and the mercury finally placed in the incubator for a short time to ensure dryness. For very exacting work the mercury, after cleaning and drying, may be distilled. Pyrex or similar resistance glassware must be used for this purpose.

Other Methods of Cutting Glass

(1) For cutting ordinary flat glass the glazier's diamond is obviously the best. A completely flat surface upon which to lay the glass, and a sharp solid edge across which to " break " the glass along the scratch marks made are both essential For ordinary straight cuts, the glass is pressed firmly down on the flat surface and a rigid straight edge (metal rule held down by a length of wood or similar object) placed along the line at which the glass is to be cut, not forgetting to allow a small margin for the thickness of the metal end of the diamond-cutter, and the cutter drawn sharply along this edge. The work should be inspected to make sure that the entire length is scratched, leaving no gaps, and that the scratch extends to the edges of the glass. With the scratch mark uppermost, the glass is moved until the scratch is exactly parallel with the sharp edge of the bench and the bulk of the glass still flat on the bench. The overhanging portion of the glass is gripped firmly and jerked downwards when the glass should break evenly along the scratch mark.

(2) To make a clean cut round a large-bored tube (or flask-neck, etc.), a scratch is made completely round it as on page 35 and a hot wire drawn tightly round the mark. The wire may be heated in the blowpipe flame or by an electrical current passed through it after it is in position.

A Standard Dropper

Where it is required to add " volumes " of a series of solutions to large numbers of tubes, as in the Wassermann, Kahn, Widal and similar reactions, a specially designed dropper may be employed. Droppers regulated by screw-clips have

the disadvantage of needing re-adjustment each time they are used.

The dropper (Fig. 9) consists of a container, a float and a U-tube passed through a cork which is supported on a glass rod fixed through another cork fitted into the neck of the float.

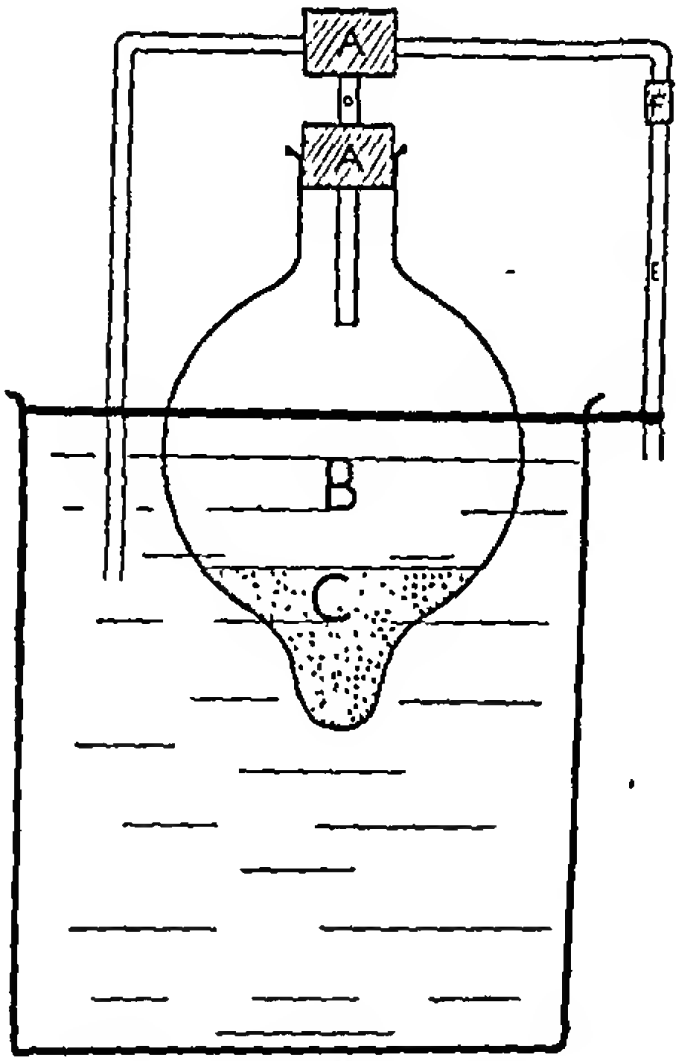


Fig 9

CONSTANT VOLUME DROPPER

A Corks.
B Float
C Lead Shot In wax.
D Glass Rod
E Standard nozzle
F. Rubber connection

The float is made from a discarded separating funnel sealed off just above the tap and part-filled with lead shot and paraffin wax. The rate of flow of drops can be regulated by sliding the glass rod up or down. A vessel such as a beaker or large evaporating dish is stood immediately below the nozzle of the dropper

so that no wastage occurs and the dropper need not be disturbed by frequent starting and stopping whilst in use. To start the dropper a length of rubber tubing is attached to the end of the nozzle and the fluid drawn over the U-tube, which then acts as a syphon. Having regulated the rate of flow it merely becomes a matter for counting the required numbers of drops falling into each tube as a rack containing tubes is passed under the nozzle of the dropper, surplus drops falling into the beaker or dish. To stop the dropper it is necessary only to lift the float until the U-tube is free of the fluid.

To make standard nozzles

With the aid of a fine carborundum stone, nozzles delivering drops of known volume can be made. Pieces of glass tubing (6 to 7 mm. diameter) are drawn out, the tapered parts cut off at points to form nozzles of varying diameters. In turn, these nozzles are attached to the dropper and drops of fluid are counted as they fall into a small graduated measuring cylinder (10 ml.).

Example.

It is required to make a nozzle that will deliver a drop of N/saline solution measuring exactly 0 1 ml.

With the nozzle to be adjusted we find that 120 drops are required to fill the cylinder to the 10 ml mark.

Then 0 1 ml. will equal 1·2 drops. A slightly larger drop is therefore required

Wet the carborundum stone and gently grind the end of the nozzle away until a slightly wider part of the taper is reached. Check delivery frequently during the process, never counting less than 100 drops.

It is most important to remember that standardized nozzles must be kept exclusively for the particular fluid for which they are originally designed as specific gravity, viscosity, etc., of the fluids are deciding factors in the volume of drops delivered.

CHAPTER VI

UPKEEP OF LABORATORY STOCK

STOREKEEPING is in itself a specialized trade and the busy technician cannot be expected to include the maintenance of an elaborate system of storekeeping in his duties. Nevertheless, it is necessary to the smooth and efficient running of the laboratory, not only to maintain a steady supply to meet the requirements of the laboratory, but to have adequate check upon consumption, expenditure, breakages and replacements.

To do this it is essential that a small room be set aside as a storeroom which should be locked and one member of the staff only held responsible for the issue and replacement of stores. This arrangement should be strictly adhered to and except where emergencies compel otherwise, no other member of the staff should have access to the storeroom. The question of ordering or actual purchase of materials cannot be dealt with here as it will depend entirely upon the system adopted for individual laboratories and, anyway, is usually a matter for "office staff" work. Whilst some may purchase direct, others merely order through central buying channels, just stipulating the commodity required and the possible source from whence it may be obtained. Whatever the system, a complete schedule of equipment, apparatus, drugs, sera, etc., should be kept.

Any one of a number of schemes can be adopted, but never "make-do" with odd exercise books or small notebooks; "make a job of it" from the beginning, something that will serve for a long time and not require frequent copying out of the entire schedule.

Scheme 1. Card Index System

In the writer's view this is definitely the best and has many advantages over ledger-books. Card indices are flexible and additions, extractions and replacements can be made

without disturbing the continuity of the alphabetically ordered system. Essential information that can be included on the cards varies slightly according to the commodity concerned. For example:

In all instances it is necessary to know—

(*a*) Source of supply.

(*b*) Cost.

(*c*) The amount of the previous order.

(*d*) Total amount in stock at any time.

(*e*) The amounts issued into use from time to time.

The specimen card below will give information on these points almost at a glance.

Specimen Card

Supplier Messrs. R Smith, Birmingham. Price 5s per 500 grms

ANILINE (A.R)

Order No	Amount received	Amount issued	Stock	Date
A762	500 grms		500 grms	4/5/46
		250 grms.	250 ,,	5/6/46
A834	500 ,,		750 ,,	6/6/47
		500 ,,	250 ,,	9/7/47
A992	500 ,,		750 ,,	23/7/48
etc				

By this we can see that the laboratory has purchased 1,500 grms. of A.R Aniline at a cost of 15s during approximately two years. Consumption has been 750 grms. during that period and there is in stock 750 grms.

A similar system can be adopted for apparatus. With more or less permanent pieces of equipment such as microscopes and other non-expendables the card can be in the form of a history-card For example:

Specimen Card

Supplier: Messrs. Jones, Ltd., London, W.1. Cost: £76. Date of purchase: 4/5/40.

MICROSCOPE: Stand No. [illegible]
and iris diaphragm, mech[illegible]
$\frac{1}{8}$ (No. ....), $\frac{1}{12}$ (No. ....

Order No.	Date sent		Cost	Date returned
A665	8/3/46.	Repair to substage	£3 10s.	10/9/46
A789	11/6/47.	1/12th lens polished	£2 2s	12/11/47
etc.				

When maintaining records of such perishable items as sera, etc., it becomes necessary to have a record of dates of expiry and a separate index is needed for these. It can be devised as follows:

Supplier: XYZ Standards Laboratories. Cost: £1 2s 6d per 10 ml.
S. paradys. (polyvalent serum) titre 1:250.

Order No.	Amount received.	Expiry date	How disposed of	Date
A456	5 ml.	10/3/47	into use	5/2/47
A557	5 ml.	11/8/47	discarded	4/1/48
A687	5 ml.	4/12/47	into use	4/1/48

When placing perishable items into stock the expiry dates must be clearly marked on the shelves on which they are kept so that easy reference can be made. For this purpose small metal card-holders can be fixed to the shelves into which cards bearing the required information can be slipped. When adding to stocks of chemicals, existing stocks should be brought to the front of the shelves and new stocks stored behind them so that all materials are used in correct sequence.

Scheme 2. Ledgers

A good stout book that will stand up to considerable "thumbing" and provide ample room for entries over long

LEFT-HAND PAGE: RIGHT-HAND PAGES—AND ALL CUT PAGES (BOTH SIDES).

Item	Sched. amt	Supplier	Cost	Reference	Amt instock	Reference	Amt. instock
Chemicals							
Acetic acid	2 w q	B.D.H.	7s. 2d. w.q.	Recd 2 w.q. (12/3/46)	2 w.q.	Into use 1 w q. (3/5/46)	1 w.q.
Acetone	2 w.q.	B D.H.	6s. w.q.	Recd. 2 w q. (6/6/46)	2 w q	Into use 1 w.q (8/6/46)	1 w q.
Bromine (2 ml. amps.)	48	Smith (B'ham)	6s. 6d. doz.	Recd. 48 (7/6/46)	48	Into use 12 (9/7/46)	36
Benzene	1 w q	B D H.	3s 9d w.q	Recd. 1 w.q.	1 w.q.		
Calcium lactate	250 g.	B D H	2s. 3d. 250 g		250 g	Into use 100 grms (4/5/46)	150 grms.
etc.							
Glassware							
Beakers (2-litre)	6	" X " Coy., London	7s 6d each	Recd 4 (4/4/46)	8	Into use 3 (4/4/46)	5
etc.							

periods should be obtained in order to avoid the laborious task of copying out fresh lists too often.

The "headed" lists of materials together with sources of supply and a "schedule amount" of each should be entered on the left side of a page; the following two or three pages should then be cut so that the lists of items and schedule amounts are visible when these cut pages are turned over. The left-hand side of the next complete page is then used for continuing the list or for entering the next "headed" list of items. A further two or three pages are cut as before and so on throughout the book. Double columns are then ruled on the remaining space available on all pages across both sides of the cut pages and to the edge of the right-hand side of the next complete page. The two sections of each double column are then headed "Reference" and "Amount in Stock" respectively. As stores are issued or received an appropriate entry is made in the "reference" column, whilst the amount remaining in stock is recorded in the "Amount in Stock" column. Incidentally, orders for further supplies should be made as soon as the stock is reduced to approximately half of the schedule amount.

At the end of each "headed" list of items ample provision should be made for entering additional items. These will not, with the ledger system, be in alphabetical order and must remain so until a new ledger is eventually compiled.

Stocktaking, under either system, becomes a comparatively simple matter, in fact, provided that care is exercised with entering and withdrawing stores, the cards or ledger will give the exact position of stock at any time. Periodic checks of the records against the actual quantities in stock are advised "*just in case . . .!*"

Breakage chits

Where a number of technicians are employed, a "breakage chit" system is recommended. Not so much as a check upon individual breakages, but in order that a rapid survey of the "apparatus position" of the laboratories can be made when stocktaking. Chits such as the following give all the

necessary information required and can be filed for reference at the time that stocktaking is carried out.

" .. .. .. Laboratory "

" .. . Department "

ACCIDENTAL BREAKAGE CERTIFICATE

Item. . .. .

Cause of breakage . . . .

Date . Signed .

CHAPTER VII

LABORATORY RECORDS OF SPECIMENS

As specimens are received at the laboratory they must, obviously, be recorded in a general register. This entails the introduction of a system of numbering. In large busy laboratories these numbers become somewhat unwieldy towards the end of each year, leaving an opening for errors such as the transposition of figures when numbering the specimens. Whilst it is difficult—almost impossible—to entirely eradicate the risk of this occurring, the number of figures used can be considerably reduced by using the letter of the month as a prefix.

Example

January	J/1	J/2	J/3	and so on.
February	F/1	„	„	„
March	M/1	„	„	„
April	A/1	„	„	„
May	My/1		„	„
June	Ju/1	„	„	„
July	Jy/1	„	„	„
August	Au/1	„	„	„
September	S/1	„	„	„
October	O/1	„	„	„
November	N/1	„	„	„
December	D/1	„	„	„

Most laboratories make use of a standardized form upon which details of the patient, clinical history, nature of the specimen and the test required, together with space for writing the report are included. Such forms should accompany their respective specimens throughout and be returned to the office only when reports are completed.

At each bench a " Specimen " diary should be kept in which the serial number, nature of specimen and date upon which the report is completed are entered. Such diaries should be treated with respect and not allowed to become " dog-eared " and illegible.

When reports pass back to the office they should be filed in order of serial numbers and typewritten copies of the reports sent out. All that is then entailed is the following:

(a) Office register with pages ruled and headed as :

Date	Serial No	Patient's name	Sender	Report File No
July 1st	Jy/21	R Jones	Dr Brown	8/48
,,	Jy/22	K Thomas	Dr. Green	8/48
,,	Jy/23	B. Black	"X" Hospital	8/48
etc.				

(b) Bench Diary :

Date	Serial No	Nature of Spec.	Test required	Reported
July 1st	Jy/21	Sputum	? T B	July 3rd
,,	Jy/22	Stool	Gen. culture	July 7th
,,	Jy/23	Blood	Widal	July 2nd
etc.				

(c) A standardized form for specimens (adaptable) :

Date. . . . Serial Number. . .. .
Patient's name . . .. .
Address . ..
. . . . .

Sender's name .. ..
Address . .. .
....

Serial No. of previous report . .. .
Nature of specimen . . . . .
Examination required . . .

Date received at the laboratory . . .. . .

CLINICAL HISTORY OF CASE

For laboratory use only Date . . ..

REPORT
(continue overleaf if necessary)

Entered in diary . . Signed .. . ..
(initials of technician) *Pathologist*

The slight trouble involved in cross-reference from diary to register to file, if and when it may become necessary, is more than compensated for by the elimination of much unnecessary duplication of writing out reports with its consequent risk of wrong reports being given.

CHAPTER VIII

LABORATORY ANIMALS

FOR the average Bacteriological laboratory breeding animals is generally considered scarcely worth the trouble, equipment and staff involved, and animals are bought in large or small *quantities as required* from the various professional breeders who specialize in supplying laboratory animals. Yet, for ordinary routine work, where " pure " blood stocks and other refinements are not essential, there is no reason why a steady supply of home-bred healthy mongrels should not be maintained. In any case the important question of adequate housing, cleaning, and feeding have to be considered wherever laboratory animals are kept.

The first consideration is, of course, the position of the animals' quarters. However clean and well cared for, each species of animal has its characteristic odour and all laboratory animals should, for that reason alone, be housed well away from the laboratory proper, preferably in an outhouse or draught-proof shed.

The floor of the animal house should be of brick or concrete and should have central gullies running the entire length of the floor of each compartment; these should lead away to a drain fitted with a trap to prevent blockages caused by wisps of straw and other oddments. The two halves of each floor should slope slightly towards these gullies.

Open stock-pens at floor level for guinea-pigs and rabbits have the advantage of being easily cleaned by ordinary scrubbing and flushing with weak disinfectant, followed by swilling with clear water. Along each side of the central gullies sufficient space should be left for a path affording easy access to cages

Assume, for example, that the only accommodation available for animals for a particular laboratory is a single-roomed outhouse. Then the central gully must divide the " healthy " *from the " infected "* animals and the house must be designed accordingly. (See Fig. 10, page 53.)

One side of the house, reserved for healthy stock, can be divided into a series of pens and all that is required is a quantity of timber of 1½-in. section, some cement, ½-in. gauge galvanized chicken-netting (2 ft. wide) and some nails.

Make a row of holes, approximately 4 in. deep by 3 in. in diameter, 2 ft. apart and at least 2 ft. from the central gully along the entire length of the room. From the timber, cut

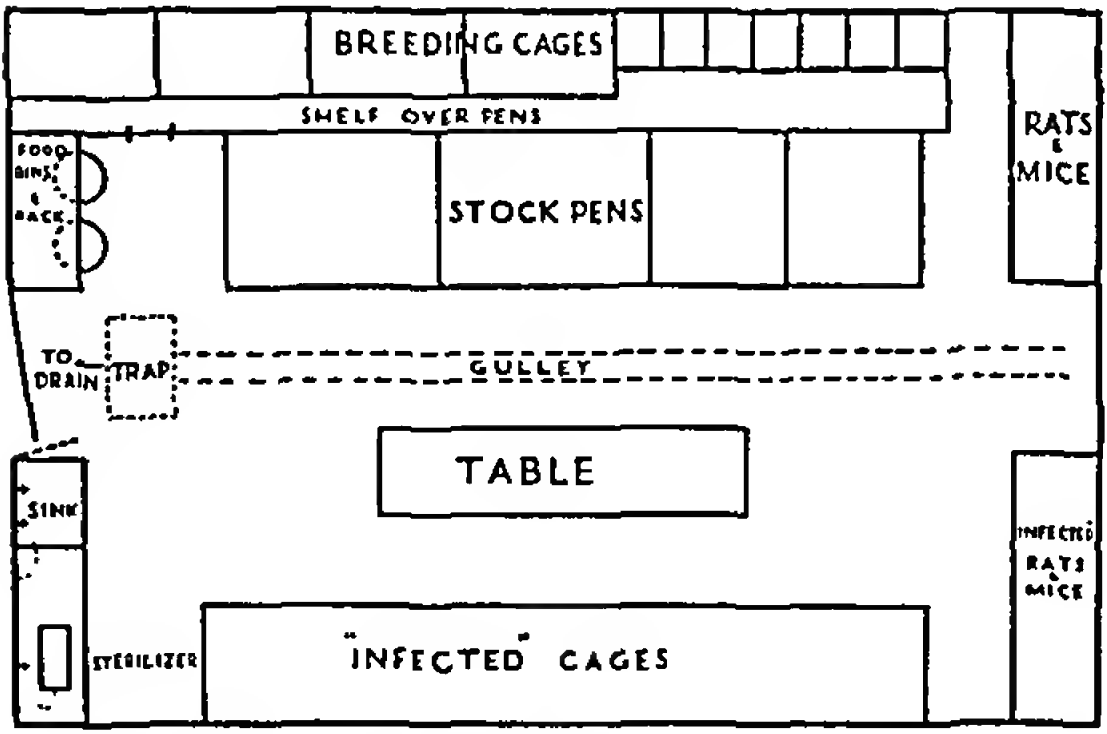


Fig 10

Suggested lay-out for a single-roomed animal house, assuming space available is an outhouse 18 feet x 12 feet (see page 52)

lengths measuring 2 ft. 4 in., and, after creosoting the ends, insert them into the holes, cementing them firmly in (these "uprights" will need support until the cement has hardened). Next cut lengths of timber as follows: (NOTE.—Follow the lettering on Fig. 11, page 54):

One length measuring 1 ft. 11 in. (A) in respect of each upright. One

Nail one end of (B) to the top of (A) and the *side* of the other end of (A) to one end of (C) Bore two holes in the plaster between the bricks of the rear wall and insert wood blocks so that 4-in nails driven through (A) will hold it in position with its lower end 1 in above the floor Next nail

the *side* of one end of (B) on to the top of the upright. Cut appropriate lengths of galvanized netting to enclose the areas bounded by the "upright", (A), (B) and (C) for each pen. Cut two lengths of the timber the entire length of the fronts of the pens and nail one along the tops of the uprights and the other 1 in. above floor level along the bottoms of the uprights, driving the nails through both the front strip of wood and the upright into the ends of (C). Cover the fronts also with netting

We now have a series of pens which can be easily flushed without risk of the woodwork becoming fouled by droppings, urine or cleaning water. There is no point in making elaborate gates to the pens, it is an easy matter to step over the netting.

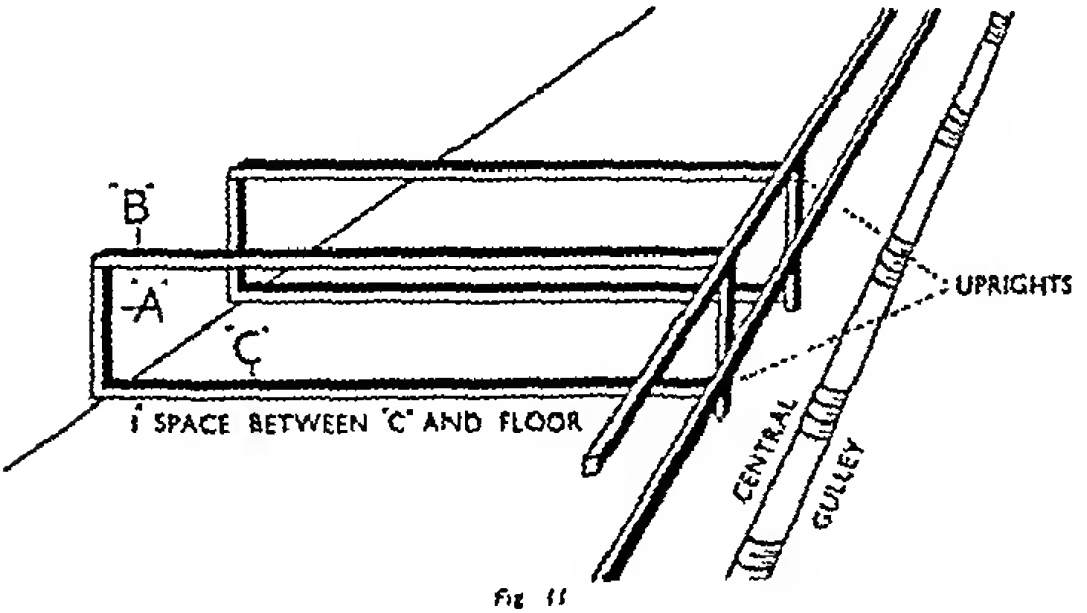


Fig 11

Where rabbits are housed, a loose wire-net cover can be made for each pen. A liberal sprinkling of sawdust (changed frequently) with a handful of clean straw in one corner of each pen will supply all the "home comforts" needed to keep the animals warm and contented.

A shelf, about waist-high, running the entire length of the same wall can be used for "breeding" cages. This shelf should have a strip of galvanized iron sheeting along its edge and protruding above the shelf for about 1 to 1½ in. to prevent foodstuff and excreta dropping into the pens below. The galvanized iron strip should terminate about 1 ft. from one end of the shelf so that the shelf can be swept to this end and the sweepings scraped into a bucket held beneath the opening.

Breeding cages can be erected in the form of a double or triple row of wooden cubicles each measuring, for guinea-pigs 1 ft. × 9 in. × 9 in., and for rabbits 2 ft. 6 in. × 1 ft. 6 in × 1 ft. 6 in. Each cubicle should be fitted with a wire-mesh door and a removable shallow metal tray covering the entire floor-space. The series of cubicles can be made after the style of shop-fixtures (rather like those seen on the walls of many grocery shops). This is a great saving in timber as the dividing partitions then form both ceiling and floor for upper and lower cages and the upright partitions one wall for each of two cages.

A similar shelf or shelves can be fitted at one end of the animal house for cages to hold mice and rats. All-metal cages are a distinct advantage for these rodents as their habit of nibbling any wooden structure within reach soon destroys the stoutest of timber cages. The remaining half of the room can now be devoted to animals which have become infected or are under observation following experimentation.

Rows of cubicles as already described may be fixed on shelves along the rear wall of this half of the room. A narrow wooden bench, the top covered with galvanized sheeting or zinc, along the centre of this half of the room will prove invaluable when removing animals for examination or performing autopsies.

The remaining wall, let into which is, presumably, the entrance door, can be utilized for racks to contain green food-stuffs and food-bins standing on the floor beneath the racks. The racks are best made from lengths of chicken-netting loosely stretched between brackets fixed to the wall. By this means the green food will be kept well drained and aerated. Ordinary lidded galvanized dust-bins are ideal for storing dry foods such as bran and oats, safe from the attentions of any visiting rodents. All foodstuffs must, of course, be kept on the "healthy" side of the gully. The remaining part of the wall on the "infected" side can be used to fix the sink and tap, and for hanging a length of hose-pipe.

Whilst the foregoing example is restricted to a one-roomed animal house the general principles of layout may be adopted for multi-roomed premises.

Feeding laboratory animals

Apart from special dieting which will be specifically detailed for individual cases that may be under observation, feeding laboratory animals is a simple enough matter. Through over-affection for one's charges, a common fault is over-feeding and frequently, in consequence, the animals become fat and somewhat lethargic.

For rabbits the equivalent of four large cabbage leaves and a liberal handful of a mixture of equal parts of bran and oats is sufficient for one day's rations; whilst for guinea-pigs the equivalent of two large cabbage leaves and a somewhat smaller handful of mixed bran and oats will prove ample for the same period of time. The green food should be varied occasionally with carrot, swede, turnip or the outside stalks of celery. Raw potato should never be given.

Regarding the supply of water to rabbits and guinea-pigs!—by the manner in which these creatures drink when water is given them during hot weather, it seems that a restricted supply at all times does no harm and is certainly appreciated by the animals. A large medicine bottle, clamped to the side of the cage with its neck inverted into a shallow enamelled bowl will provide a supply of clean water.

Despite popular belief, cheese is not the staple diet of rats and mice! Stale bread, soaked unpolished rice, bran and oats mixed with small quantities of green foods will keep these rodents well fed and contented. An occasional morsel of cheese or a small quantity of cod liver oil will provide all the fats they require. Rats and mice definitely require a regular supply of water. An ordinary medicine bottle, fitted with a cork through which passes a short length of capillary glass tubing about 3 mm. bore (making quite sure that the end of the tubing is well rounded off) is clamped to the side of the cage in an inverted position. The animals very quickly learn to obtain a drink by licking the end of the tubing and there is no risk of the floor of the cage becoming wet from overturned drinking vessels.

If undertaking breeding, it is most satisfactory to keep males and females segregated and mating selected couples in the

breeding cages; removing the male after a few days and leaving the female to produce and rear her " family ".

General points to be considered

1. *Cleanliness.* The cages should be cleared of old sawdust and straw regularly each day. At least once each fortnight all stock animals should be removed to wooden boxes or similar temporary quarters whilst the entire floor is washed with weak disinfectant and swilled with clear water. The floor must be allowed to dry thoroughly before returning the animals to their pens.

2. *Removing animals from cages.* By providing a wire cover which will fit into the metal trays of cages, animals complete with their metal trays can be removed from the cubicles whilst the woodwork is cleaned. A few extra trays will enable animals to be transferred to fresh trays whilst those in which they have been living are thoroughly disinfected and cleaned and laid aside to take their turn as replacements

3. *Feeding vessels.* Great care should always be exercised to see that animals receive their own feeding vessels after cleaning, etc. This is, obviously, of particular importance on the " infected " side of the house.

4. *Labelling.* Each " infected " cage should be fitted with a small card-holder in which to keep the animal's case-card. This card should have full details regarding the animal plainly written on it, including its age, original weight, date of inoculation, inoculum (or whatever experiment is being performed), and any other relevant details concerning it. If the back of the card is subsequently used to record the outcome of the experiment, post-mortem findings, etc., the card can be filed as a permanent record of the " case " from start to finish.

5. *Animal house walls.* If not tiled, the walls of the animal house should be limewashed regularly.

6. *Inspection of stock.* A frequent inspection of all stock-pens should be made for signs of illness amongst the animals. A close examination of the excrement will quickly reveal any abnormality and would automatically lead to closer inspection of individual occupants of the pen. All suspected animals must be immediately segregated. Their subsequent fate will

Ulceration of the genitals. This is caused by an organism similar in appearance to Treponema pallidum (T. cuniculi). Do not attempt to breed from suspected animals.

Rats

Feeding. Once per day only. Approximately 2 oz. of food is given.

Breeding. Gestation period is 21 days. Young ones are weaned at 6 weeks. Breeding animals should be rested a further two weeks after weaning a litter before breeding again.

Common diseases. Intestinal infections. Usually caused by Salm. enteritidis. Very infectious and suspects must be immediately segregated.

Mange. This usually affects the ears and root of the tail. This is a common disease and must be watched for regularly It appears as a grey warty condition and can be treated by applications of an ointment made up of:

Sulphur (resublimed)	2 parts
Sodium carbonate	1 part
Lard or vaseline	16 parts.

Mice

Feeding. Approximately 1 oz. of food per day.

Breeding. Gestation period is 18 to 21 days. Young ones are weaned at four weeks. Females are rested for a further two weeks before re-breeding.

Mice reserved for breeding should be given a small amount of canary-seed or millet occasionally.

Not more than three litters should be produced from any one pair of animals.

Common diseases. Intestinal infections (Salm. enteritidis).

Infectious ectromelia. This disease is a virus infection and may take one of two forms: Acute (necrosis of liver and spleen); Chronic (enlargement of one foot with subsequent oedema and gangrene). In both cases the animals must be destroyed immediately and the remaining stock carefully watched for at least two weeks for further outbreaks. Destruction of the entire stock may be necessary. Cages must be completely sterilized before introducing fresh stocks.

T[illegible] are [illegible] of the mammary carcinomata.

CHAPTER IX

ANIMAL POST-MORTEM TECHNIQUE

WHERE possible, a table suitable for post-mortem work, provided with gas and water supplies, can be included in the "infected" section of the animal house and all post-mortems carried out there. (See Fig. 10, page 53.)

General equipment required:

(*a*) A selection of scalpels (Swann Morton type is recommended).
(*b*) Scissors: blunt and sharp pointed (small and medium-sized).
(*c*) Forceps: blunt and sharp pointed.
(*d*) Small bone forceps.
(*e*) Probes.
(*f*) Razor.
(*g*) Searing-iron (a small square of copper sheet welded to a rod in a wooden handle).
(*h*) Fish kettle sterilizer.
(*i*) Shallow enamelled-iron dissecting dishes with metal or wood trays which fit inside them.
(*j*) Small shallow glass dissecting dishes (8 in. × 4 in. × 2 in. approximately), half filled with black dissecting wax.
(*k*) Rubber gloves and apron.
(*l*) Enamelled buckets, with lids.
(*m*) Large dissecting pins (2 in. × $\frac{1}{16}$ in.) and pinning-out awls.
(*n*) Sterile cotton-wool swabs and small squares of lint.
(*o*) Lysol and 1:1,000 solution perchloride of mercury.

Incidental requirements (according to work on hand):

(*a*) Sterile petri-dishes.
(*b*) ,, pipettes and teats.
(*c*) ,, screw-cap pots (approximately 4 oz.).
(*d*) ,, test-tubes.
(*e*) Various media.
(*f*) Small jars containing formol saline solution.
(*g*) Grease pencil.

Preparing animals for autopsy

Preferably the whole exposed parts of the chest and abdomen of the animal should be shaved. This step, whilst obviously an added precaution against contamination, is not essential and unless the technician is skilled in the use of the ordinary "cut-throat" razor, should be omitted as there is grave risk of penetrating the skin and possibly *introducing* contamination. The washings will drain off into the dish. Small squares of lint soaked in 2 per cent. lysol or 1:1,000 perchloride of mercury are placed over the animal's head and lower extremities.

Guinea-pigs and rabbits. Lengths of string are tied securely to the legs, using half-hitch knots, and each length passed through a perforation near each corner of the metal tray, or if using wooden trays through a small hook screwed into the wood near each corner, pulled taut and tied to the other loose end of string in the knot. Alternatively wood-handled pinning-out awls may be used for pinning the limbs directly to the wood. The tray is placed in the dissecting dish and the animal well swabbed over with cotton-wool swabs soaked in weak lysol (2 per cent.).

Rats and mice. Using the large dissecting pins the animal is pinned out on the black dissecting wax in a glass dish.

Autopsy. A long incision is made through the skin *only* of the chest and abdomen from the neck to the pelvis and further incisions made across the centre of the two halves thus made.

The four "sections" of skin are laid or pinned back, leaving the muscles of the chest and abdomen clearly exposed. Similar incisions are next made in the abdominal wall, care being taken to avoid puncturing (*a*) the diaphragm where it is attached to the ribs, (*b*) any organs in the abdomen. These sections of the abdominal wall are laid back, exposing the contents of the peritoneal cavity. The organs may be removed as required, remembering to change instruments frequently and taking particular care to avoid puncturing any part of the alimentary system. The lower protruding part (xiphisternal cartilage) of the sternum is held firmly with forceps whilst

the ribs are cut through along a line following the diaphragm to the 9th rib where the tissues connecting this to the 10th rib are cut, outwards, towards the side of the animal. The scissors are then turned towards the neck and the ribs cut through, up to the neck, along the side of the animal. This is repeated on the opposite side and the V-shaped piece thus severed is laid back over the animal's head. Sterile pipettes fitted with rubber teats may be used to remove any fluid from the thoracic cavity or for puncturing the heart for removal of blood, should samples of these fluids be required for further investigation. Such organs as may be required for further investigation are placed in sterile petri-dishes as they are removed. Tissues for "section" are placed in the small pots of formol saline solution and carefully labelled. Upon completion of the autopsy 5 per cent. lysol solution is poured liberally over the remains and the four strings cut (or awls withdrawn) and the contents of the tray consigned to the furnace. The dishes with the trays are then sterilized and washed.

Should there be any possibility of infection from animals, rubber gloves and apron should be worn.

Instruments, with the exception of scalpels, should be boiled in distilled water in the fish-kettle for at least twenty minutes, after which the tray holding the instruments is removed and placed on a square of lint soaked in 1:1,000 perchloride of mercury. The scalpels may rest, blade downwards, in small jars containing methylated spirit and with a small pad of lint or cotton-wool at the bottom.

Separate instruments should be used for each stage of the autopsy, and, in consequence, a reasonable supply should be available. Instruments should not be returned directly from use back into the sterilizer as this coagulates protein matter adhering to them, making cleaning difficult and staining the metal. All instruments as they are finished with during the autopsy should be placed in a shallow dish containing 20 per cent. lysol solution.

CHAPTER X

STERILITY AND STERILIZATION

THE word "sterile" indicates freedom from all living matter, whilst the word "aseptic" indicates freedom from harmful or disease-producing bacteria

Some confusion is frequently experienced with regard to the true meaning of the various words employed when discussing sterilization, disinfection and kindred subjects. Whilst opinions continue to differ, the writer ventures the following reasonable assumptions:

Disinfectant. A substance which will completely destroy vegetative forms of bacteria which are capable of producing harmful effects (either in the form of actual disease or the decomposition of matter).

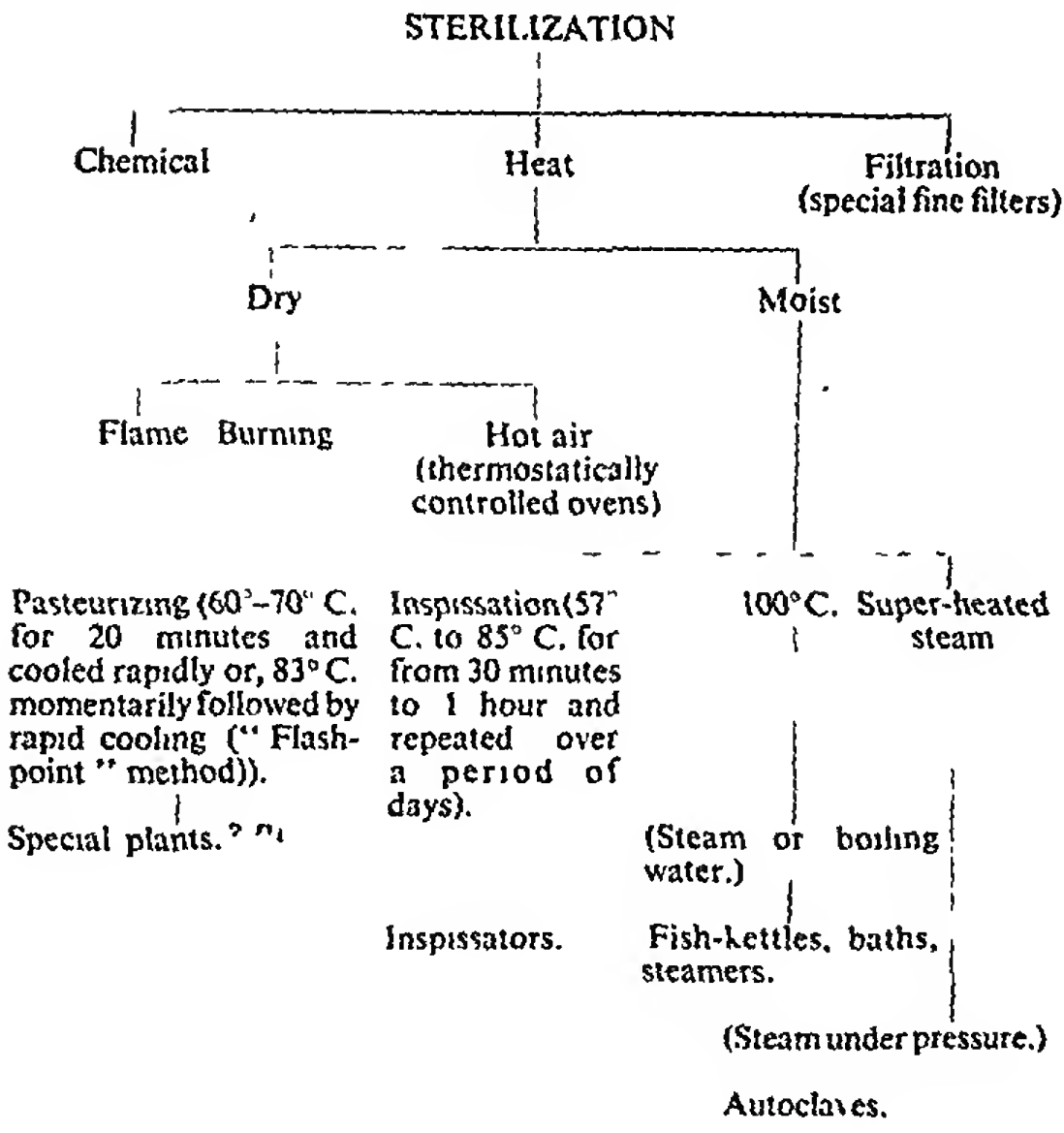
Antiseptic. A substance which will inhibit the action of "sepsis-producing" bacteria either by killing them outright or arresting their growth. The meaning of the word—"against sepsis"—indicates such a substance as being harmless to tissues when used for the purpose of arresting infections of wounds, etc., hence we see advertised "antiseptic" ointments and lotions.

Germicide. A substance capable of destroying vegetative forms of bacteria whether harmful or otherwise.

All the foregoing are closely interrelated as much depends upon the strength of solution of the substance used, length of time of exposure of the bacteria to its influence and so on.

For example, a strong solution of phenol can be both Germicide and Disinfectant, whilst a weak solution of the same substance can be used as an antiseptic lotion.

Chemical sterilization has a limited field of use in the Bacteriological laboratory, an extremely useful one nevertheless! This method is mainly used for discarded cultures (followed by



autoclaving as an added precaution!), personal protection, preservation of substances and for some metal instruments.

Heat is the most frequently used method of sterilization, particularly MOIST heat.

Boiling water is used for sterilizing syringes, instruments, needles and some rubber apparatus.

Steam at 100° C. is used extensively for the sterilization of culture media. Fractional sterilization (or Tyndalization) is usually employed when using steam at 100° C. and this involves heating the material to be sterilized for twenty to thirty minutes on each of three successive days (see "The Steamer", page 68).

uperheated steam. When using superheated steam it is

to speak in terms of " atmospheres " or " pounds-per-
e-inch pressure ". For ordinary purposes atmospheric
ure is taken to be 15 lb. per square inch, so that if material
be sterilized at *One Atmosphere* it means 15 lb per square
over and above ordinary atmospheric pressure. (See
e Autoclave ", page 67.)

pissation. This form of sterilization is employed when
aterials to be sterilized may be damaged by any greater
or the bubbling which accompanies boiling may leave
ishes in a medium which is coagulated by heat (See
e Inspissator ", page 69.)

steurization. This is a method of " incomplete " steriliza-
employed largely in processing foods, milk, etc, and is
m used in the laboratory.
r sterilization by filtration, see special chapter, page 181.

ming. This is employed mainly at the work bench
tubes, etc., are opened. The mouth of the vessel is
d through the bunsen flame to kill any organisms resting
ie rim which might find their way into the vessel.

rning. This more or less speaks for itself. Platinum
s and needles are always " burned off " to red heat in the
en flame both before and after touching living cultures.

ot air. Sterilization by hot air is carried out in an oven
ially constructed for the purpose Most glassware,
ided it is thoroughly dried prior to being placed in the
, may be sterilized by this method 160° C. to 180° C.
½ hours is required. (See " Hot-air Ovens ", page 69.)

The Rideal-Walker Test (Phenol coefficient)

1903 Rideal and Walker introduced a method for com-
ng the germicidal power of substances of phenol-like
istency with that of pure phenol.
Many modifications of the test have been introduced and its
e has extended to substances widely differing from phenol,
in the essentials it remains the same
he important point to bear in mind is that the comparison
t be made under identical conditions.

These conditions are:

(*a*) Time that the bacteria are exposed to the action of the germicides (phenol and unknown).
(*b*) The presence and quantity of organic matter.
(*c*) Temperature at which the test is carried out.
(*d*) The bacteria used for the test.
(*e*) Age of the culture of bacteria used.
(*f*) Length of time that subcultures are incubated and the temperature.
(*g*) Constituents of the medium used.
(*h*) Reaction of the medium and fluids used.

Ruehle and Brewer add to these:

(*i*) Size of the test-tubes used (150 mm. × 25 mm.).
(*j*) Quantity of fluid in the tubes (5 ml.).
(*k*) The organism used must be subcultured daily for five successive days prior to use and a 24-hour broth culture of the organism used.

Technique

5 ml. quantities of a series of dilutions of phenol and the unknown germicide are measured into the standard test-tubes and placed in a water-bath at 20° C. where they are allowed to remain for at least five minutes to adjust the temperature with that of the bath. The 24-hour broth culture is shaken and placed in the bath for fifteen minutes to allow any clumps of bacteria to settle. 0·5 ml. of the culture is measured into each tube and, at intervals of five, ten and fifteen minutes a loopful (4 mm. diameter) is transferred to a corresponding tube of broth (10 ml. quantity). The " transfer " tubes are incubated for forty-eight hours at 37° C. (or longer if bacteriostasis is suspected). Where certain metals are involved the subcultures are made into 100 ml. quantities of broth to obviate the known strongly bacteriostatic action of these metals.

The phenol coefficient is calculated by dividing the highest dilution of the unknown germicide to kill the test-organisms in ten minutes but not in five minutes, by the dilution of phenol to give a corresponding result.

Example

Germicide	Dilution	Time of exposure of organism in minutes		
		5	10	15
Phenol	1 50	−	−	−
	1 60	−	−	−
	1 70	+	−	−
	1.80	+	+	−
	1 90	+	+	+
	1 100			
Unknown Germicide	1:125	−	−	−
	1 150	−	−	−
	1 175	+	−	−
	1:200	+	+	−
	1.225	+	+	+

As the resistance of different bacteria to germicides varies, the name of the organism used is included when results are recorded.

Assuming that S. typhosa was used in the example given, then the germicide would be said to have a S. typhosa phenol coefficient of 2·5.

The autoclave

A form of sterilizer for moist-heat sterilization at temperatures above that of boiling steam at atmospheric pressure (100° C.). It consists of a vertical or horizontal cylinder in an outer casing. The cylinder is fitted with a lid which is clamped tight by means of wing-nuts. Instruments for recording temperature and pressure within the cylinder are also fitted (usually in the lid). Important points to bear in mind when using the autoclave are: Open the air-release tap when the lid is first closed and do not close the tap until all the air is expelled and steam issues freely from it. (The temperature of a mixture of steam and air at a given pressure is less than that of pure steam.) Set the safety-valve to "blow-off" at the required pressure and count the time for sterilization from the time the pressure required is reached. Do not open the autoclave until

the contents have cooled at least to below 100° C. (or the pressure gauge has returned to " zero "). Opening the autoclave before this point will cause liquids to boil violently when exposed to ordinary atmospheric pressure as they will still be at a temperature above that at which water ordinarily boils. The superheated steam within the autoclave must have free access to all non-liquid materials which are to be sterilized; it is useless to seal, say, some dry asbestos pads, in a steam-tight container before placing it in the autoclave. The degree of effective heating would be that of the same temperature in a hot-air oven. The main advantage of the autoclave is that sterilization is completed in one period of heating (for most routine work this is one atmosphere—121·6° C. for thirty minutes). The autoclave is limited in its use for the preparation of culture-media because many of the constituents of media, particularly carbohydrates, are destroyed or altered in composition by excessive heating.

Table of pressures with equivalent temperatures

Pressure in lb.	Temperature	
	Centigrade	Fahrenheit
5	107 7	227
10	115·5	240
15	121·6	250
20	126·6	260
25	130·5	267
30	134 4	274

The steamer (Koch and Arnold)

This form of sterilizer is designed to work at the temperature of boiling steam at atmospheric pressure (100° C.). As many bacterial spores are able to resist this temperature for long periods, FRACTIONAL STERILIZATION (or Tyndallization) is the usual procedure. The material to be sterilized is heated at 100° C. for twenty minutes after which it is cooled and allowed to remain at room-temperature for twenty-four hours when it

is again heated for a further twenty minutes. A third heating is given after a further twenty-four-hour interval. The principle of this is that spores which have resisted the primary and secondary heating will develop into vegetative forms during the twenty-four-hour intervals and, as such, will be destroyed during subsequent heating.

The advantage of this method of sterilization for culture media such as those containing carbohydrates or gelatin is that twenty minutes at 100 °C. is insufficient to decompose the carbohydrates or to interfere with the solidifying properties of the gelatin. The method is not entirely efficient as spores which are present in materials unsuited for development of vegetative forms remain unaffected. It so happens, however, that most such substances, distilled water and similar non-nutrient fluids, may be autoclaved anyway.

Hot-air ovens

This form of sterilizer is very restricted in its use, as (*a*) it is only efficient at temperatures of 160° and over, (*b*) penetrating power of hot air is far less than that of steam at much lower temperatures, (*c*) culture media cannot be sterilized in it under any circumstances. The apparatus consists of a double-walled oven, the outer wall usually lagged with asbestos to minimize loss of heat by radiation, thermostatically controlled, fitted with a thermometer and a "damping" device to control the flow of air through it when in operation. Its use is confined to the sterilization of laboratory glassware (which must always be perfectly dry before placing it in the oven). Cotton-wool, corks (other than rubber) will withstand a temperature of 160° C. for 1½ hours. Care should always be taken when placing apparatus in the oven to avoid touching the metal sides of the oven with cotton-wool plugs or corks.

The inspissator

An oven-type of sterilizer with a water jacket and sloping racks fitted inside, used for the preparation of solidified serum or egg-medium "slopes". In order to avoid disruption of

the surface of such slopes by steam-bubbles rising in the medium as it coagulates, the temperature of the inspissator is never taken beyond 85° C. Fractional sterilization (see "The steamer" above) is essential. Both gas and electrically heated models are manufactured. The best type for general use is an electrically heated apparatus with a water-jacket which permits of free steam entering the inner chamber where the medium is placed. Should cotton-wool plugs be used for the tubes, the medium will not dry up as it would in a dry chamber.

CHAPTER XI

CENTRIFUGES

MANY laboratory centrifuges are, regrettably, subjected to abuse ! quite unwittingly in most cases it is true, nevertheless, within a short time comparatively new machines develop vibration and noise. Lack of lubrication is not always the fault. Construction of the centrifuge to allow of free access to the " head " (part which holds the " buckets ") means that all bearings are below this part in such models and any slight extra weight in one bucket will cause the centrifuge to tend to spin at an angle (like an unbalanced top) causing excessive wear of the bearings. A small pair of scales, kept near the machine, upon which to balance the buckets and their contents before placing them in position will obviate much of the wear and prolong the efficient life of the centrifuge.

Most makers issue a " lubrication chart " with each machine and this chart, together with the lubricant should be kept near the centrifuge. Where machines are constantly in use a " lubricating calendar " is justified. The calendar can be just a simple card upon which the dates when the machine is lubricated are entered:

CENTRIFUGE LUBRICATED ON :

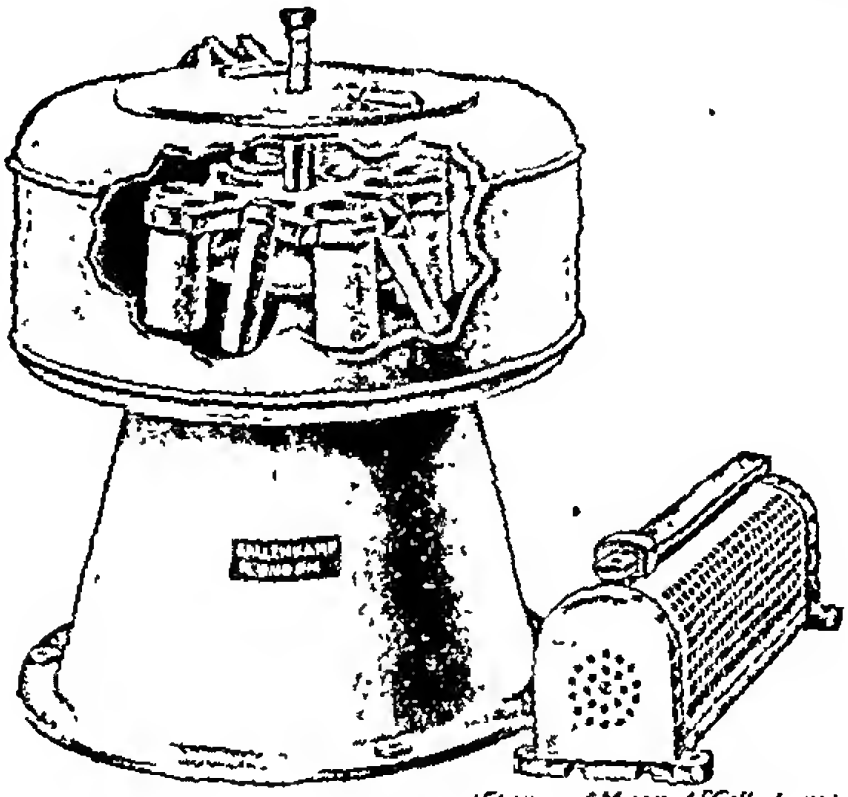
18/12/47
21/1/48
12/2/48
etc

With electric models trouble is sometimes experienced with the brushes of the motor. As the commutator is cylindrical the brushes will wear down until they fit snugly on to this and for this reason, if removing them care should be taken to see that they are replaced in the same position.

To clean the commutator, remove the brushes, insert a piece of clean soft cloth drawn tightly over the unsharpened end of a pencil and press lightly on to the commutator whilst revolving the head of the machine slowly by hand. The area

of cloth in contact with the commutator should be changed several times during the process.

Most electric centrifuges are fitted with a rheostat control for regulating speed. It is important that a set procedure for starting and stopping the machine should be adopted. Far too great a load is placed upon the motor by switching on whilst the rheostat is in any position other than at "start". Speed should be increased very gradually up to that required.



(Courtesy of Messrs. A. Gallenkamp)

Fig. 12.
An Electric Centrifuge

When stopping the centrifuge the rheostat control should be returned to the "start" position and then the current switched off. If this is made a routine procedure, the risk of burning out the windings of the motor is reduced to a minimum.

The hinged lid is something more than just a dust-cover: it should always be closed when the machine is running in order to reduce air-resistance.

Few technicians can memorize the speeds indicated by the "bubble-type" revolution indicators fitted to many of the larger models and a small chart showing the lines with corresponding speeds, pasted to the side of the machine, is a useful reminder.

CHAPTER XII

IMMUNITY AND IMMUNOLOGICAL TECHNIQUE

WHY do we recover from, or succumb to infection? and why, say, when someone suffering from a cold sneezes amongst fellow passengers in a train, some of those passengers become ill, whilst others remain unaffected? It is frequently a matter of immunity and degrees of immunity.

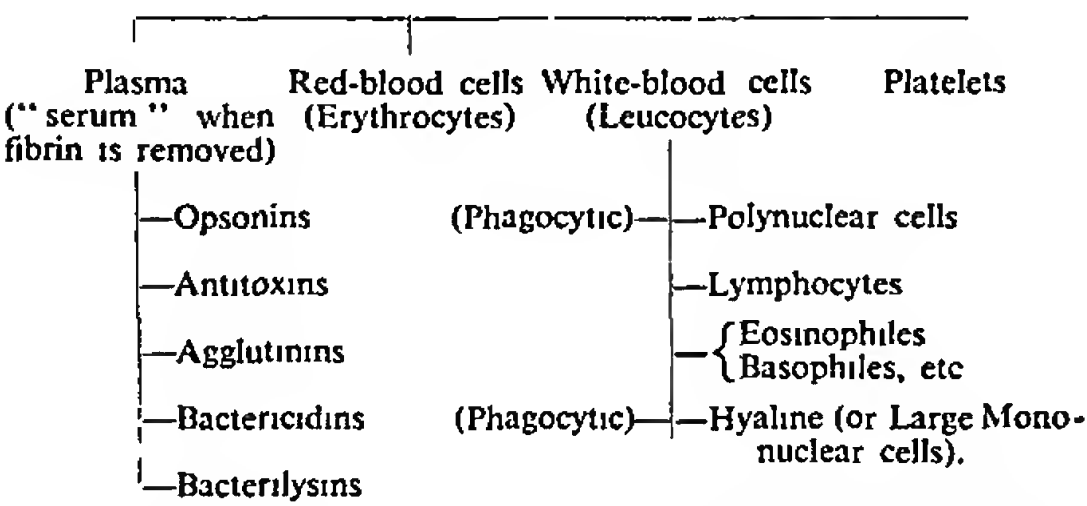
Immunity means the power to resist:

(*a*) Infection by pathogenic micro-organisms and

(*b*) The injurious effects of the toxins (poisons) they produce.

Immunity involves a whole range of activities within the bloodstream and in order that we may understand this it is necessary to know something of the constituents of the blood.

BLOOD



The constituents of the blood with which we are mainly concerned when discussing immunity are: Plasma (or serum), Polynuclear and Hyaline cells.

Phagocytosis. About the middle of the nineteenth century a Russian scientist—Metchnikoff—experimented with starfish. He observed that when he inserted granules of carmine into the tissues of the fish "strange wandering cells" swarmed towards these particles, surrounding and eventually absorbing

them. He called these cells "Phagocytes". Metchnikoff had seen, for the first time in history, one of the main defences of the body against bacterial invasion actually in operation. The polynuclear and hyaline cells are capable of attacking bacteria which enter the body and of ingesting them and dissolving them up into soluble substances which are passed away from the tissues through the normal channels through which waste products are removed. An obscure substance known as "Opsonin" must be present in the blood plasma before these cells can undertake this task. This substance acts, either upon the invading bacteria, preparing them for ingestion, or upon the phagocytes making them capable of attacking the bacteria, it is not quite definite which! That this vague substance exists is quite certain, and it can be readily demonstrated by a special test known as "Opsonic Index" which involves the mixing of active leucocytes with the serum of the person to be tested and adding to this mixture a number of the bacteria in question. After a period of time in the incubator (during which the preparation must not be allowed to dry) films are made, stained and examined microscopically. The average number of bacteria ingested by each leucocyte is calculated by counting the total numbers ingested by, say, fifty or more cells.

Antigens. All protein matter possesses what is known as "Antigenic" properties. An "antigen" is any substance which, when introduced into the tissues provokes the formation of anti-bodies which react specifically with that antigen. Now, when bacteria enter the tissues they act as "antigens", provoking the formation by the tissues of substances which attempt to destroy, or neutralize both them and the effects which they may have upon the tissues. If this reaction does not occur the invading bacteria gain the ascendancy and the tissues are destroyed. If it is delayed then the tissues become damaged. Even when the tissues *do* react to the invasion there are, inevitably, casualties on both sides and some of the phagocytes (large mononuclear or hyaline cells) have the dual task of clearing away the dead cells and attacking the invaders. The antibodies which are formed and which are carried throughout the tissues by the bloodstream, are soluble substances held in solution by

the blood-plasma and other body-fluids. These substances are capable of one or more of the following functions:

(*a*) Causing invading bacteria to clump together (Agglutinins).

(*b*) Neutralizing poisons produced by the bacteria (Antitoxins).

(*c*) Killing the bacteria and leaving them to be ingested by the phagocytes (Bactericidins).

(*d*) Dissolving the bacteria completely (Bacterilysins).

The Widal and Similar Reactions

The phenomenon of the agglutination of bacteria by specific antibodies (agglutinins) present in the blood-stream of humans and animals suffering from the disease caused by those bacteria or artificially inoculated with them, can be put to practical use in the laboratory. The amount of agglutinins present in the blood can be measured and, in consequence, the degree of reaction to the bacteria can be gauged. The reaction can be used in two ways, either an unknown serum can be tested against a known organism or an unknown organism against a known serum. When examining an unknown serum a uniform suspension of the known organism is used and progressive dilutions of the serum mixed with a measured volume of the suspension of organisms. The highest dilution of serum which causes the organisms to clump together (agglutinate) is said to be the titre of that serum.

Before testing unknown organisms against a known serum, the titre of the serum is first established by titrating it against the organism for which it is specific. It must be carefully borne in mind that a number of organisms may possess one or all of three types of antigen-" H " (Flagellar) said to be contained in the flagella of the organism; " O " (somatic) in the body of the organism and " Vi " associated with the degree of virulence of the organism. Continued cultivation of certain bacteria in artificial media or under adverse conditions causes loss of virulence with consequent loss of the last antigenic characteristic. For routine diagnostic work standard cultures and specific sera are obtainable commercially, but for special work great care must be exercised in the selection of suitable known strains of organisms.

The agglutination test in most common use to-day is, perhaps, the Widal Reaction for Typhoid and the para-typhoid infections and standard suspensions of the organisms of each and the specific sera for all three phases—" H ", " O ", and " Vi " are obtainable.

Technique similar to that for the Widal Reaction can be applied for testing many organisms and the specific antibodies in serum. For making the suspensions, formalized cultures are usually used, but in certain instances, when " O " agglutination is sought, as formalin interferes with this antigen, living or alcoholized cultures are employed. It is essential that the greatest possible care is exercised when carrying out tests with living cultures and ready means of sterilization for all apparatus used must be at hand as well as precautionary measures taken against infection of the worker.

Methods for carrying out the test

1. *Dropping.* Standardized dropping-pipettes (Dreyer's pipettes) are used. This technique may be employed for any particular organism against a known serum or vice versa. Small test-tubes measuring 3 in. × ½ in. are used for diluting the serum and standard Dreyer agglutination tubes for the actual test. A quantity of N/saline, racks to hold the tubes, a small sterilizer containing boiling water and a grease pencil complete the apparatus required.

Technique. A 1:10 dilution of the serum is made in a small tube by adding 10 drops of serum to 90 drops of N/saline. Using a separate pipette for each, drops of dilute serum, N/saline, and bacterial suspenson are added to a row of Dreyer tubes as follows:

Tube No.	1	2	3	4	5
1:10 serum dil.	10	5	2	1	0
N/saline solution	0	5	8	9	10
Bacterial Suspension	15	15	15	15	15
Resultant Dilutions	1:25	1:50	1:125	1:250	control

This procedure can be repeated for a number of organisms or strains of the same organism or for a particular organism against varying sera, care being taken always to label rows of tubes clearly.

A further series of dilutions may be made by diluting the 1:10 serum dilution a further ten times (i.e. 10 drops of 1:10 serum added to 90 drops of N/saline) and proceeding as before. It will be noted that, in this case, the first tube of the second series will correspond to the last tube of the first series. This has the advantage of being a check upon the previous tube (the degree of agglutination should be the same in each).

2. *Measuring* Graduated 5-ml. pipettes (graduated in tenths) and 1-ml. pipettes (graduated in hundredths) fitted with short lengths of rubber tubing with glass mouthpieces are used. The serum dilution is made in a 5 in. × $\frac{5}{8}$ in. test-tube and the Dreyer-tubes are set out as for Method No. 1. 4·75 ml. of N/saline is measured into the test-tube and, using the 1-ml. pipette, 0·25 ml. of serum added (making a dilution of 1:20). Using separate pipettes for each, N/saline, dilute serum and bacterial suspension are added to a row of five tubes as follows:

Tube No.	1	2	3	4	5
N/saline	0	0·5	0·5	0·5	1·0 ml.
1:20 serum dilution	1·0	0·5	0·5	0·5	
		Mix and Transfer ↗	Mix and Transfer ↗	Mix and Discard	→
Bact. suspension	0·5	0·5	0·5	0·5	ml.
Resultant dilutions	1:30	1:60	1:120	1:240	control

In tube 2 the contents are thoroughly mixed following the addition of the dilute serum, and 0·5 ml. of the mixture transferred to tube 3. The contents of this tube are mixed thoroughly and 0·5 ml. of the mixture transferred to tube 4. Again the contents are mixed and 0·5 ml. from this tube is

either discarded or kept in a small tube for the next series of dilutions.

As before, this procedure can be repeated for a number of organisms or strains of the same organism. In this instance, however, by making further dilution of the 1:20 serum by adding 1 ml. to 9 ml. of N/saline we will have a 1:200 dilution giving a second series dilutions of: 1:300, 1:600, 1:1,200, 1:2,400, and a control. Alternatively, the 0·5 ml. of diluted serum from tube 4 of the first series may be set aside for continuing the progression dilutions (1:480, 1:960, 1:1,920, and 1:3,840).

Slide agglutinations. It is sometimes expedient to attempt an agglutination reaction with isolated colonies from plate cultures. This is fraught with grave risks, particularly as (*a*) substances used in the preparation of certain selective media interfere with the antigenic structure of the bacteria and agglutination will not occur under these conditions and (*b*) bacteria in certain phases of growth (i.e. when "rough" colonies are produced) are deficient in the antigen which characterizes them, or they may auto-agglutinate (produce a degree of clumping without the addition of antiserum). Slide agglutinations can be performed (A) where results may be a matter of urgency, provided that the organism is grown on simple nutrient media, (B) when typing organisms such as streptococci, pneumococci and meningococci against type-specific sera as in Griffith's method for typing streptococci, and (C) where agglutination occurs very rapidly and there is little risk of drying over a period of time. Fairly thick emulsions of the organism to be tested are made in N/saline and added to a small amount of specific serum on a micro-slide. The slide is then gently rocked to and fro, causing the drops of mixture to run across the surface of the slide, keeping the organisms agitated in each of the mixtures.

Example. An unknown organism is to be tested against a series of specific sera. Using a grease pencil or the end of a micro-slide dipped in hot vaseline, divide a micro-slide up into a series of "cells" as in Fig. 13. Using a large platinum loop and sterilizing it between each stage of the operation, place a loopful of one of the specific sera into the first "cell", the next serum into the second "cell" and so on, leaving the end "cell" free to contain the control suspension of the

organism. Add a loopful of suspension of the organism to each " cell " and a loopful of N/saline to the control.

Great care must be taken to see that the mixtures do not flow over the grease-barriers into adjoining cells. Examine with a hand-lens for evidence of agglutination, comparing each with the control. Evidence of agglutination, if present, should eventually become visible to the naked eye.

Should it be required to titrate an unknown organism against a specific serum up to the exact end-point of the titre of the serum, equal quantities of serum-dilution and suspension of the organism may be used. This means that to each tube,

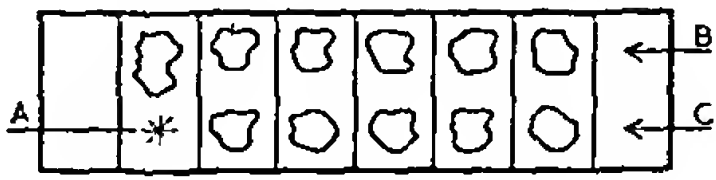


Fig 13.

A "—N/saline
" B "—Bacterial suspensions
" C "—Specific sera

diluted serum of just half the final dilution will be added prior to the addition of the suspension and, working on this basis *the serum dilutions in the tubes may be calculated backwards* from the last tube of the series.

Example.—The maximum titre of a specific serum is, say, 1 in 250 Then, the fourth tube will contain, prior to adding the suspension of organisms, 1:125 dilution of serum and each of the remaining tubes will contain:

Tube No.	1	2	3	4	5
0·5 ml. of serum dilution of	1:15 125	1:31·25	1:62 5	1:125	control
0 5 ml. of bact suspn.		—added to each tube—			
Final dilutions	1:31·25	1:62·5	1.125	1.250	control

Dilution of small quantities

There are times when quantities of fluids to be diluted are so small that even the " dropping " method is impracticable. Provided that a fairly high dilution is required which

will result in a reasonably large volume of the ultimate dilution, the difficulty may be overcome with the aid of a graduated pipette (either 0·1 ml. or 1·0 ml. according to the amount involved) and an ordinary capillary pipette fitted with a rubber teat. As an illustration of the technique to be employed let us assume that 0·8 ml. of a 1:40 dilution of serum is required.

E.g.—0·2 ml. of N/saline is drawn into a 1·0 ml. graduated pipette and the pipette laid on its side on a level surface with the point just clear of the edge. The column of fluid must remain at the tip of the pipette

[illegible] mall
[illegible] ding
[illegible] the
[illegible] the

glassware is clean and free from grease the serum will run into the graduated pipette until the column of fluid reaches the 0·22 mark.

The capillary pipette is washed out with two or three washings of N/saline and then, with it, N/saline is run into the graduated pipette until the column of fluid reaches the 0·8 mark. The tube destined to hold the diluted serum is slid over the end of the graduated pipette and both the pipette and tube are raised into the perpendicular position, gently blowing the diluted serum into the tube.

Paraffin-slides

Where minute quantities of fluids are to be used, "paraffin-slides" upon which to make dilutions, and capillary pipettes fitted with a throttled-control (see page 39) may be employed.

To make the paraffin-slides cut a small square of lint and tie it tightly over one end of a slide, leaving a smooth edge for spreading the paraffin-wax. Clean paraffin-wax (m.p. 60° C.) is heated to 120° C. in an evaporating basin and a number of clean slides laid out on blotting paper on the bench. The lint-covered slide is dipped in the molten wax and rapidly drawn lengthwise over the surface of a slide leaving a thin even film of wax. Re-dip the spreader for each slide. Allow the slides to cool and avoid touching the waxed surfaces, which should be practically sterile. Place the waxed slides together, waxed surface to waxed surface, in bundles of six pairs. With the lint covered slide cover all four sides of each bundle with wax, sealing them completely.

When required for use, a pair of slides may be prized off by inserting the point of a scalpel or penknife and levering them off the bundle.

The slides may then be laid, wax surface uppermost, on the bench and minute measured volumes of fluid expelled on to them without fear of the drops spreading or running together.

Water-baths used for agglutination reactions

Practically all agglutination reactions performed in tubes are heated in water-baths to a temperature of 56° C. It is not so much the heating as the setting up of convection currents within the tubes that is of importance. For this reason baths should never be filled beyond a point where the water reaches half-way up the tubes when racks are placed in the bath. Distilled water should invariably be used for filling baths; this is particularly important where "O" and "Vi" agglutination reactions are involved as the clumping of the organisms is of a granular character and traces of deposit from impure water on the outer surfaces of the tubes can lead to confusion. If using a bath without a lid, a glass sheet should be fitted over the bath as illustrated below.

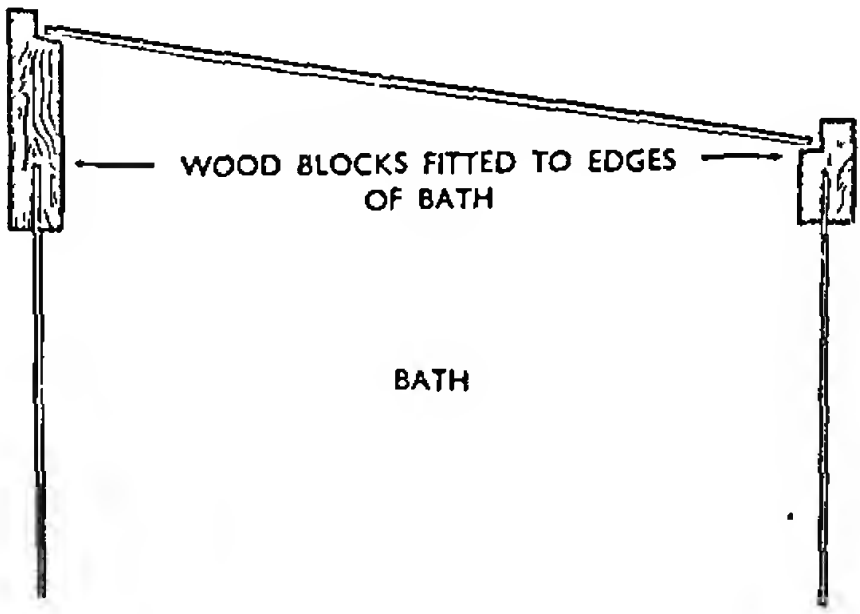


FIG 14

Common antigens

An important feature of certain groups of organisms (i.e. the Salmonellae group) is the possession by members of the groups of a "common antigen". For example: a serum which agglutinates S. schottmulleri will also agglutinate S typhmurium. In order that such organisms may be correctly

classified it becomes necessary to perform "absorption tests" which involve saturation of a group-specific serum with the organism under investigation and then testing that serum against standard strains of each of the organisms in the group for which the serum is specific. The standard strain which produces no further agglutination corresponds to the organism under investigation. Or, by adding a thick emulsion of a 24-hour agar culture of the organism to be tested to a dilution of a specific serum (at least sixty times stronger than its known titre) and incubating the mixture at 37° C. for four hours, then centrifuging the mixture at high speed and separating off the serum into a fresh tube. A series of dilutions of this "absorbed" serum is set up as for an ordinary agglutination test (remembering that it is already diluted) and equal volumes of a thin suspension of the standard organism for which the serum is specific are added. If no further agglutination is evident the organism under investigation is identical with the standard organism; if agglutination does occur then the two organisms are different. Similar technique to the above may be employed to absorb the common antibody from a "group" serum leaving the specific antibody for subsequent titration.

Whilst standard specific sera are usually obtainable commercially, preparation of agglutinating sera can be carried out in the laboratory. Healthy rabbits of not less than 4 lb. (approximately 2,000 grms.) in weight are selected and their serum tested against the salmonellae organisms which commonly infect rabbits to ensure that immune bodies are not already present. Twenty-four-hour agar slope cultures of the organisms involved are emulsified in a measured amount of N/saline and killed by immersion in a vaccine-bath at 60° C. for one hour. At weekly intervals doses of this emulsion are injected intravenously, commencing with 1/20th of the bulk and doubling the dose; one half of the original culture eventually being given. Where very toxic organisms are used the commencing dose should be much less. Where anti-serum containing "H" or "O" antibodies only are required, great care must be taken to ensure that selected strains of organisms possess the necessary characteristics, or that the cultures are suitably treated to destroy the unwanted antigen (i e. formolized

cultures should be used for producing "H" antibodies, whilst cultures should be treated with alcohol for producing "O" antibodies).

Antibodies present in the animal's blood should be at their highest titre seven to fourteen days after the final injection, when a sample of blood may be withdrawn from an ear-vein and the serum separated off and tested against the organisms used. Agglutination should occur in a dilution of at least 1:1,600.

The animal may be bled either from a neck-vein or by cardiac puncture and the blood allowed to clot. The clot should be left in the cold (preferably in the refrigerator) overnight after which the serum is removed and 0·5 per cent. phenol added. (NOTE.—Para-chlor meta-cresol or glycerine may also be used as a preservative.) The serum can then be bottled off in 1·0 ml. or 5·0 ml. quantities and stored in the cold. Alternatively the serum may be dried *in vacuo* by one of the following methods.

Hartley's method. The serum is placed in shallow, flat-bottomed dishes in a vacuum desiccator containing freshly fused calcium chloride or phosphorus pentoxide The desiccator is connected to a vacuum pump (Geryk or Hyvac) and a vacuum maintained to within 1 to 2 mm. of mercury from complete. The serum should freeze solid within twenty minutes, but water vapour will continue to be given off and be absorbed by the calcium chloride or phosphorus pentoxide until, within a few hours, the serum is completely dried.

Craigie's method. Previously cooled serum is poured into shallow dishes (petri-dishes without lids) to a depth of 3 mm. The dishes are supported on corks, which act as heat-insulators, in a large desiccator containing freshly fused calcium chloride. The desiccator is connected to a vacuum pump and vacuum maintained for twenty minutes The tap of the desiccator is then turned off, the pump disconnected and the desiccator placed in the refrigerator. The serum should be completely dried within twenty-four hours. The dried serum is stored in small screw-cap bottles enclosed in a large vessel containing calcium chloride, or in small glass ampoules sealed in the blowpipe flame (great care being taken to avoid heating the dried serum in the process). When required for use, one part of dried serum is added to fifteen parts of sterile distilled water.

CHAPTER XIII

THE WASSERMANN, KAHN, AND SIMILAR REACTIONS

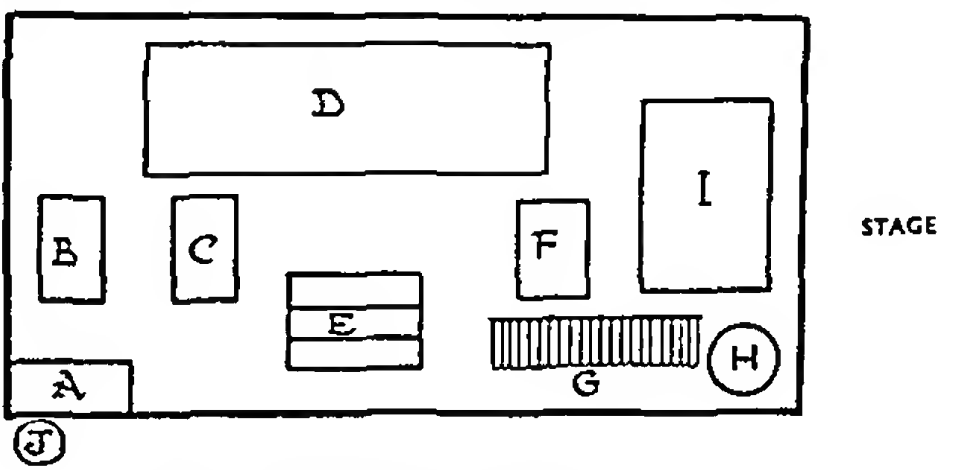
MANY methods of performing these tests have been devised and examples only are given in the following text. The general techniques are essentially the same ; the amounts and dilutions of reagents being the main varients.

The Wassermann Reaction

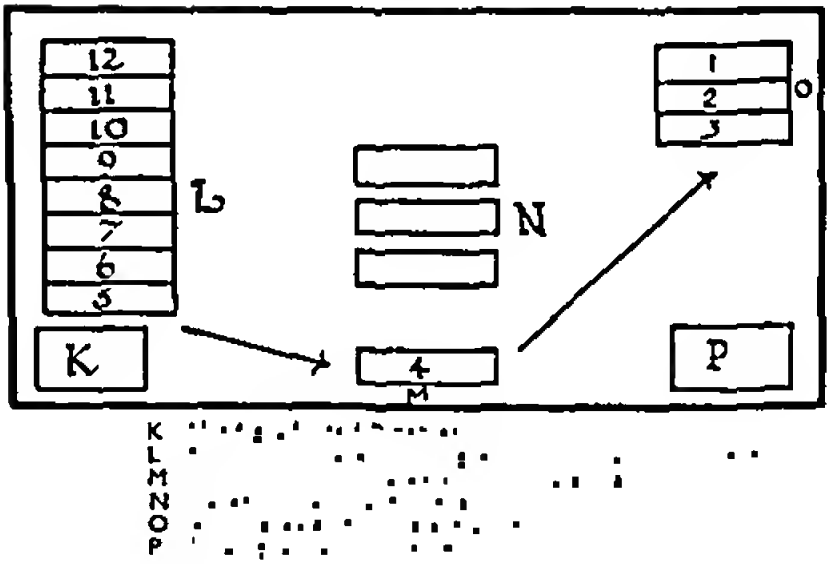
This reaction is used in the diagnosis of syphilis and resembles other complement-fixation tests except that the "antigen" used is not, in the true sense of the word, a *real antigen* (i.e. antigen protein derived from the causative agent of the disease) but a colloidal suspension of substances including alcoholic extracts of muscle-tissue and cholesterol.

Separating sera from blood-samples. (See diagram at foot of page.)

Provided that standardized collection tubes are used for blood samples, these may be centrifuged as they arrive and the



A Scales for balancing Centrifuge-Buckets
B Used Serum Pipettes (in jar of water)
C Clean Serum-Pipettes
D Racks of Blood-Samples (numbered)
E WR Racks for Sera
F Serum Tubes
G Serum Tubes (numbered)
H Glass-Ink and Pen
I Inactivation Bath
J Centrifuge (on floor)



R Antigen suspension
S Complement dilution
T ½ strength complement for controls
U Antigen/complement mixture
W Position of WR Racks when Reagents are added
X WR Baths
Y Hæmolytic system

Fig 15

A SCHEME FOR BENCH LAY-OUT FOR THE WASSERMANN REACTION

clear serum transferred to labelled tubes in a Wassermann rack ready for inactivation. The sample-tubes are numbered consecutively from No. 1 onwards for each batch of bloods examined and placed out in rows of twenty at the back of the bench. The serum tubes are similarly numbered. A good supply of short-nozzled pipettes about 6 in. long with capillaries of no more than 1 in., together with a number of rubber teats should be available. The clean pipettes are kept in a box at the front of the bench, whilst those which have been used are dropped into a dish containing water, at the rear of the bench to one side of the blood-sample racks.

Preparation of antigen

Required:

Apparatus	*Materials*
Glass pestle and mortar.	Heart-muscle (human or sheep).
Glass powder.	
Filter papers (Whatman No. 1).	Abs. alcohol.
	Ether meth.
Glass-stoppered bottles.	

Technique. Human (or sheep) heart is freed from all fat and the left ventricle muscle cut into small pieces. The muscle is then weighed and absolute alcohol added in the proportion of 9 ml. to each gram of muscle by grinding small amounts of muscle at a time with glass powder in the mortar and adding small quantities of absolute alcohol to each "grinding". The mixture is placed in a glass-stoppered bottle together with the remainder of the alcohol, in the incubator at 37° C. for twenty-four hours. The bottle is shaken vigorously every hour during the working-day. A Whatman filter paper, previously washed in ether and allowed to dry, is placed in a large funnel and the alcoholic suspension of muscle filtered through it into a fresh bottle. This is best done in the refrigerator overnight (the use of a large funnel and paper enables the worker to pour the entire volume of alcoholic suspension into the funnel). The filtrate is re-filtered the next day, stoppered tightly and allowed to stand at room temperature for three weeks.

Preparation of cholesterol solution

1 per cent. cholesterol in absolute alcohol is incubated until the cholesterol is completely dissolved. 1 per cent. of this solution is added to the antigen.

Haemolytic serum (amboceptor)

Erlich applied the term "Amboceptor" to the obscure substance in a serum sensitized against a specific antigen which reacts specifically with the antigen. The "Amboceptor" used in the Wassermann reaction is a serum which has been sensitized against the red blood corpuscles of sheep and is

generally known as haemolytic serum. The serum is best obtained commercially.

Complement

Freshly-killed guinea-pig serum, "Lyovac" (a commercial product), or dehydrated guinea-pig serum (as for agglutinating sera, see page 83) may be employed according to individual choice. If using fresh serum, small male guinea-pigs or non-pregnant females must be used. After anaesthetizing, the guinea-pigs are bled, by cutting across the throat with a sharp razor, into shallow dishes (petri-dishes) and, after cutting the clot into small segments, the dishes are placed in the 37° C. incubator for ten minutes. They are then transferred to a cool place for a further fifteen minutes to allow the clot to shrink rapidly, giving a good yield of serum. The serum must be free from red-blood corpuscles and should be centrifuged to ensure this.

NOTE.—Human blood also contains complement, but as it varies with individuals and as each serum to be tested in the test-proper would, in consequence, have a different amount present, the patients' own complement is destroyed by heating the patients' sera to a temperature of 56° C. for 30 minutes. The guinea-pig complement is standardized before inclusion in the test-proper (see pages 90 and 91).

Washed sheep's red-blood corpuscles

Freshly defibrinated sheep's blood is mixed with an equal volume of N/saline in centrifuge tubes and a mark made on the side of each tube at the level of the mixture. The tubes are centrifuged at 3,000 r.p.m. for twenty minutes and the supernatant fluid removed The tubes are then filled to the mark with fresh N/saline and the contents mixed thoroughly by pressing a clean rubber bung over the mouths of the tubes and inverting them several times. They are then centrifuged again, this time at 3,000 r.p.m. for ten minutes (the fluid in which the cells are now suspended is much less dense than at first and they will separate out more readily). This washing process is repeated, discarding the supernatant each time, for at least three washings or until the supernatant is water-clear. If traces of haemolysis persist after repeated washing the blood should be discarded and a fresh sample obtained.

The strength of suspension of red-blood corpuscles varies according to individual tastes of workers, but a 5 per cent. suspension in N/saline is frequently employed. This means that 5 ml. of the centrifuged deposit of cells are made up to 100 ml. with N/saline.

Standardization of the red-cell suspension

It is advisable, in view of the probable variance between samples of blood used from time to time, to standardize the suspension of red cells so that this becomes a more or less constant factor throughout all tests performed.

Method A. (*Estimation of the percentage of haemoglobin.*)

Using 0·25 ml. instead of the 0·2 ml. usual with Haldane's haemoglobinometer, the "percentage" of haemoglobin present in the 5 per cent. suspension of cells is estimated. For a 5 per cent. suspension this should be 70 per cent. Adjustment may be made by addition of N/saline or cell suspension.

Example.—100 ml. of 5 per cent. suspension is prepared.
The percentage of haemoglobin is found to be 86.
Then to make the percentage 70: $\frac{86 \times 100}{70} = 122{\cdot}85$.
So that 22·85 ml. of N/saline is added to the bulk of the suspension.

Method B. (*Estimation of the number of cells.*)

An ordinary Neubauer haemocytometer may be used.

0·1 ml. of the suspension is made up to 5 ml. with N/saline, a drop of the dilution run into the counting chamber of the haemocytometer and counted as for an ordinary blood-count.

Example.—The calculation is
Area of chamber × dilution × depth of chamber
or 400 small squares × 50 × 10
[illegible] cells are counted
[illegible] of cells per c.mm.
of the suspension.

Having established this as a standard, suspensions used in subsequent tests should approximate to this.

The above figures are an assumption for a 5 per cent. suspension; if using a 3 per cent. suspension the count would be proportionately lower.

Titration of haemolytic serum (for No. 1 method of the M.R.C.)

In a series of twelve test-tubes dilutions of haemolytic serum are made as follows:
1:10, 1:20, 1·40, 1:60, 1:80, 1:100, 1:150, 1:200, 1:300, 1:1,000, 1:1,500, 1:3,000, and from each of these, in turn, commencing with the highest dilution one volume is measured into a corresponding set of Wassermann tubes each containing one volume of R.B.C. suspension. These tubes now have the same amount of R.B.C. suspension but varying amounts of haemolytic serum. Each tube is shaken immediately after adding the serum and the set of tubes incubated for fifteen minutes at 37° C., shaking the tubes at intervals. The tubes containing 1:10 to 1.300 serum are placed aside at room temperature. *To each of the remaining tubes one volume of N/saline is* added, followed by one volume of 1·10 complement dilution. The tubes are again shaken and placed in the 37° C. bath for thirty minutes, shaking at intervals as before. The tube containing the least amount of haemolytic serum showing haemolysis represents the Minimum Haemolytic Dose (M.H.D.) of the serum. A serum giving a reading of lower than 1:1,000 should not be used in the test-proper. The 1·300 tube is examined for evidence of agglutination of the cells In the test-proper 6 M.H.D. is used, therefore a dilution of haemolytic serum giving 12 M H.D. is made and mixed with an equal volume of double strength suspension of cells.

Titration of haemolytic serum (for McIntosh and Filde's method)

A 1:1,000 dilution of the unknown haemolytic serum is prepared by adding 9·9 ml of N/saline to 0·1 ml. of the serum and, to 1 ml. of this dilution adding 9·0 ml. of N/saline. A series of nine Wassermann tubes are set up:

Tube No.	1	2	3	4	5	6	7	8	9
1:1 dil. of complement	0·1	0·1	0·1	0·1	0·1	0 1	0·1	0·1	0·1
1 1,000 dil haemolytic serum	0·1	0·2	0·3	0·4	0·5	0·6	0·7	0·8	0·9
N/saline	0·8	0·7	0·6	0·5	0·4	0·3	0·2	0·1	0·0

0·5 ml. of suspension of washed R.B.C.s is added to each tube and after shaking, the rack is placed in the 37° C. bath for sixty minutes.

The first tube showing complete haemolysis equals the M.H.D.

Example.—The 0·0005 tube showed complete haemolysis. Then as 4 M.H.D. is required for the test-proper, this means that 0·002 ml. of the original serum would be required to sensitize each 0 5 ml. of the suspension of R.B.C.s.

Titration of complement (for No. 1 method of the M.R.C.)

Using a capillary pipette graduated to deliver a standard volume (0·11 ml. is a convenient volume) a dilution of 1:10 complement in N/saline is prepared. The same pipette is used throughout the titration.

A row of eight Wassermann tubes is set up as follows:

Tube No.	1	2	3	4	5	6	7	8
	(volumes in each case)							
1:10 comp. dil.	1	1	1	1	1	1	1	1
N/saline	2	3	4	5	6	7	8	9
Resultant dilution	1:30	1:40	1:50	1:60	1:70	1:80	1:90	1:100

Two similar rows of tubes are set up in racks labelled: (*a*) Complement only, and (*b*) Antigen added, as follows:

Row (*a*)

Tube No.	1	2	3	4	5	6	7	8
N/saline (volumes)	2	2	2	2	2	2	2	2
One volume of each of the complement dilution	1:30	1:40	1:50	1:60	1:70	1:80	1:90	1:100
Sensitized R.B.C.s (volumes)	1	1	1	1	1	1	1	1

One extra tube containing 1 volume of R.B.C. suspension plus 3 volumes of N/saline is put up as a " cell control ".

Row (*b*)

Tube No.	1	2	3	4	5	6	7	8
N/saline (volumes)	1	1	1	1	1	1	1	1
One volume of each complement dilution	1:30	1:40	1:50	1:60	1:70	1:80	1:90	1:100
1:15 antigen susp.	1	1	1	1	1	1	1	1

Row (*a*) is placed in the 37° C. bath for thirty minutes, whilst row (*b*) is left at room temperature for a similar length of time. To each tube in row (*b*) one volume of sensitized R.B.C s is added and the rack placed in the 37° C. bath for thirty minutes.

Reading results. The two rows of tubes are compared and results should show that the antigen fixes less than one dose of complement; that is, assuming that complete lysis occurs in the tube of row (*a*) containing 1:80 complement then the tube in row (*b*) containing 1:40 complement should show corresponding lysis. The lowest dilution of complement in row (*a*) to show complete lysis is the one chosen for the test-proper.

Example.—All tubes up to that containing 1/80 dilution of complement were found to show complete lysis whilst the tube containing the 1/90 dilution of complement showed incomplete lysis For the test-proper 2 M.H.D., 3 M.H.D. and 5 M.H.D. are required Then just prior to carrying out the test-proper dilutions of complement of 1.40 (2 M.H D.), 1:26 66 (3 M.H.D.), and 1.1 (5 M.H.D.) are made up. The supernatant fluid of the " cell control " tube should be colourless.

Titration of complement (McIntosh and Filde's method: modified)

A dilution of complement of 1:24 and a dilution of antigen of 1:15 are made up in N/saline. i e. 0·25 ml. of complement to 5·75 ml. N/saline and 0·5 ml. neat antigen to 7·0 ml. N/saline (the saline is added to the antigen in a straight-sided wide-mouthed jar and the mixture poured back and forth several times between two similar jars).

Two rows of nine Wassermann tubes are set up and the rows labelled (*a*) complement only, and (*b*) antigen added. Using a 1-ml. pipette graduated in $\frac{1}{100}$ths, the reagents are added as follows:

Tube No.	1	2	3	4	5	6	7	8	9	10	
Row (a):											
N/saline	0·9	0·875	0·85	0·825	0·8	0·775	0·75	0·7	0·65	0·6	ml.
1:24 complement	0·1	0·125	0·15	0·175	0·2	0·225	0·25	0·3	0·35	0·4	ml.
Row (b):											
N/saline	0·4	0·375	0 35	0·325	0·3	0·275	0·25	0·2	0·15	0·1	ml.
1.24 complement	0·1	0 125	0·15	0 175	0 2	0·225	0·25	0·3	0·35	0·4	ml.
1:15 antigen	0·5	0 5	0·5	0·5	0·5	0·5	0·5	0·5	0·5	0·5	ml.

The rack containing both rows of tubes is placed in the 37° C. water-bath for thirty minutes and the tubes shaken at least twice during this period. Next 0·5 ml. of sensitized R.B.C.s suspension is added to each tube of both rows and after again shaking, the rack is left in the bath for a further thirty minutes.

Reading results. Two and a half times the amount of complement present in the pair of tubes (Rows (a) and (b)) containing the highest dilution to show complete lysis represents the strength of complement dilution to be used in the test-proper. For easy reference the following table represents the respective dilutions of complement to be prepared for use in the test-proper.

Tube No.	1	2	3	4	5	6	7	8	9	10
	First two should not be used.		1:32	1:27	1:24	1:21	1:19	1:16	1:13	1:12·5

The antigen should fix less than one-third of the complement.

Titration of antigen

Before a fresh batch of antigen is brought into use the following tests must be carried out and carefully recorded for reference.

and placing in the 37° C. water-bath for thirty minutes. The *supernatant fluid should be quite colourless.*

The test proper (No. 1 M.R.C.)

Four tubes are used for each patient's serum tested. In the following table the reagents are listed in order of adding and the racks must be shaken after each addition.

Tube No.	1	2	3	4
Volumes of:				
Patient's serum diluted 1·5	1	1	1	1
N/saline	1	1	—	—
Complement (2 M.H.D.)	—	1	—	—
Complement (3 M.H.D.)	1	—	—	1
Complement (5 M.H.D.)	—	—	1	—
Antigen 1:15 suspension	—	—	1	1

The tubes are allowed to stand at room temperature for thirty minutes and then in the 37° C. water-bath for thirty minutes. One volume of sensitized R.B.C.s is added to each tube and the tubes again shaken. Results are read off as soon as the No. 1 tubes and the No. 4 tubes show complete lysis (usually about five minutes).

The No. 2 tubes are useful controls of the complement titration as, if this has been taken a little high, there will be a delay in lysis in most of the No. 2 tubes throughout the series of sera tested and it serves as a useful warning with regard to "border-line" cases. Whenever the test is performed both "*negative*" and "*positive*" control sera and a "cell" control tube must be included. The "cell" control tube contains 1 volume of sensitized R.B.C.s plus 3 volumes of N/saline.

When testing cerebro-spinal fluids, three additional tubes must be included, containing 2, 5 and 10 times the quantities of C.S.F. as are used for sera, whilst No. 4 tube is omitted.

McIntosh and Filde's method (modified)

Method A. Three tubes are used for each serum tested.

Tube No	1	2	3
Patient's serum	0·1	0·05	0·1 ml.
Complement (diluted according to titration result)	0·25	0·25	— „
Half-strength complement dilution	—	—	0·5 „
1/15 antigen suspension	0·25	0·25	— „

When the bulk dilutions of complement and antigen destined for distribution into tubes 1 and 2 of each serum tested, have been prepared they may be mixed in reasonably small quantities and 0·5 ml. of the *mixture* added to each tube. e.g assuming that 200 sera are to be tested, then allowing for wastage, approximately 60 ml. of each of the complement dilution and antigen suspension are prepared. 25 ml of each are mixed at a time and pipetted into the appropriate tubes of the test The amount of fixation of complement during the time occupied in pipetting is negligible. All tubes are shaken and the racks placed in the 37° C. water-bath for thirty minutes. 0·25 ml. of the haemolytic system is added to each tube, the tubes again shaken and left in the bath for a further thirty minutes.

Pipettes for measuring patient's sera (McIntosh and Filde's method modified)

Where large numbers of sera are dealt with regularly it is an economic proposition to make graduated serum-pipettes in the laboratory. 12-in. lengths of 2-mm. bore tubing (external diameter 5–6 mm.) are cut and the ends rounded off in the flame. The centre of each length is heated and drawn out into a capillary approximately 1 in. long. The tubing is cut at the centre of the capillary, making two pipettes from each length. Quantities of 0·1 ml. and 0·05 ml. of mercury are measured out into watch-glasses (using the throttled standard pipettes, page 37). With the throttled control (page 39) attached to the open end of each pipette the two

aid of a special lamp designed for the purpose. The antigen may be prepared in the laboratory (see below) or obtained commercially (" Bacto " Kahn antigen is recommended).

Materials required for the test:

Apparatus

(1) Wassermann tubes (3 in × ½ in.).
(2) Small flat-bottom jars (small ointment-pots are suitable).
(3) 1·0 ml. graduated pipettes.
(4) 0·1 ml. graduated pipettes.
(5) Capillary pipettes graduated to deliver 0·0125, 0·025 and 0·05 ml. (see note on page 103).
(6) Capillary pipettes, graduated to deliver 0·15 ml.
(7) Wassermann racks.
(8) " Flocculation " lamp (see Fig. 16, page 101).

Reagents

(1) Normal saline solution.
(2) Kahn antigen.

Preparation of the antigen suspension

As the suspension of antigen is unstable it should be prepared after all the sera to be tested have been measured into their respective tubes. The antigen should not be used until ten minutes has elapsed after mixing and should be discarded after thirty minutes has elapsed. As the amount of antigen for each serum tested is 0·0875 ml., the total volume of antigen required may be calculated by multiplying this figure by the total number of sera to be tested (not forgetting the control sera) and allowing a small amount for possible wastage during mixing and pipetting. Antigen is diluted with N/saline in the proportion of 1 : 1·1. The required amount of antigen is measured into one jar and, using a fresh pipette, the saline is measured into the other jar. The two are mixed by pouring the saline into the antigen and pouring the mixture from one jar to the other at least six times.

Test-proper. For the test-proper three tubes are set up for each serum to be tested and reagents added as follows:

Tube No.	1	2	3	
Patient's serum	0·15	0·15	0·15	ml.
Antigen suspension	0·05	0·025	0·0125	,,
Resultant dilution of serum	1·3	1:6	1:12	

The tubes are shaken, preferably in a shaking machine, for three minutes and then placed in the water-bath at 37° C. for fifteen minutes N/saline is then added as follows.

	1·0	0·5	0·5	ml.

Antigen control

Tube No	1	2	3	
Antigen suspension	0·05	0·025	0·0125	ml.
N/saline solution	0·15	0·15	0·15	,,

Shake and incubate as for test-proper and add:

N/saline solution	1·0	0·5	0·5	ml

Kahn quantitative test (applicable only to strongly positive sera)

In this test a constant volume of antigen suspension is used with varying dilutions of serum and results are expressed in terms of " Kahn units ".

Kahn unit

With complete precipitation in the tube containing undiluted serum and antigen suspension the reaction represents four " Kahn units ". If precipitation occurs in certain dilutions of serum, the potency of that serum in terms of " Kahn units " equals four times the highest dilution of serum giving precipitation.

i.e. the highest dilution of a given serum to show precipitation was found to be 1:20 then that serum contains 4×20 (80) Kahn units.

and may be interpreted by one of the three methods below.

Alternative methods of recording.

4	++++	+'
2	+++	+'
1	+	±
0	0	0

Some difficulty may be encountered when reading Kahn results, particularly with the weaker reactions. The special lamp (Fig. 16) incorporating means of illuminating the tubes by direct and indirect light and giving a magnified image of the tubes in a concave mirror whilst they are held at an angle, has been devised by the author, and serves, also, for viewing agglutination reactions.

Kahn verification test

Experiments conducted by Kahn indicate that in certain instances, positive results may be due to biological changes other than those specific to syphilis. By performing tests at 2° C. and 37° C. in parallel he was able to differentiate between syphilitic and non-syphilitic reactions. He further demonstrated that the sensitivity of the syphilitic reaction was increased at 37° C. as compared to room temperature.

Preparation of Kahn antigen (Oswald and McLean)

Fresh beef-heart, freed from fat and fibrous tissue, is finely minced, spread in thin layers on a porcelain plate and dried. The dried material is then broken up in a coffee-grinder. 50 grm. of the dried muscle is placed in an Erlenmayer flask and just covered with ether. This is stored in the refrigerator for four days, replacing the ether each day (store for a few days longer, repeating the ether-washing if, at the fourth day, the ether is not "water-clear"). The muscle is then spread on filter-paper and dried at room temperature. 20 grm. of this dried muscle is covered with 100 ml. of absolute alcohol in a stoppered bottle and allowed to remain in the refrigerator for nine days and then at room temperature for one day. The alcoholic extract is filtered off through a Whatman No. 1 paper and cholesterol added at the rate of 4 mgrm. per ml. of extract.

Making Graduated Pipettes for the Kahn Test

Using a standard " throttle " pipette (see page 37), 0·1 ml. of mercury is measured into a watch-glass. A short length of 6-mm. diameter glass tubing is drawn out into a long even-bored capillary, making sure that plenty of glass is softened to form a sturdy wall to the capillary. The capillary is cut leaving a long length for calibration. A rubber teat is attached to the wide end of the pipette and the bead of mercury drawn up into the capillary so that it is part-way up and well clear of the tip; the tip is then sealed in the blow-pipe flame A mark is made with a grease-pencil or with glass-ink at the end of the column of mercury nearest the teat-end of the pipette and the capillary cut off at the other end of the column of mercury. The teat is removed and the mercury allowed to run back into the watch-glass. Assuming that the capillary has been carefully drawn and that the bore is reasonably even, it is placed against a rule graduated in millimetres and the space occupied by the mercury measured and divided into two equal parts. Next the half nearest the tip is divided into two equal parts and the lower half of this again divided into two equal parts. The finished pipette will be capable of delivering amounts of 0·05 ml., 0·025 ml. and 0·0125 ml. all in one operation.

To operate. Fluid is drawn into the pipette up to the 0·0125 ml. mark; the pipette is lifted clear of the fluid, a small air bubble allowed to enter and the pipette dipped into the fluid and 0·025 ml. drawn into it. It is again lifted clear of the fluid, another small air bubble allowed to enter and finally 0·05 ml. is drawn in. These quantities can then be expelled, one at a time into the respective tubes used in the test. The pipette graduated to deliver 0·15 ml. is made in a similar manner, but

Fig. 17

Throttled serum pipette, with three equal volumes of serum interspaced with air bubbles.

the capillary is drawn with a slightly wider bore. The tip-end is calibrated with mercury (adding a further 0·05 ml. to that

already in the watch-glass). A succession of volumes each measuring 0·15 ml. interspaced with small air bubbles may be sucked into the capillary and expelled, one at a time, when performing the test. It is advisable, when using these pipettes to fit a " throttled control " (see Fig. 8, page 40).

Complement-fixation Test for Gonorrhoea

Antigen. Several strains of gonococci grown for twenty-four to forty-eight hours on hydrocele-agar in Rouxe-bottles are washed off with N/saline and mixed in a cylinder with 100 ml. of N/saline. 1·0 ml. of N/1NaOH is added and the whole incubated at 37° C. for two hours (this dissolves most of the organisms). The fluid is then filtered through sterile lint. The filtrate is cleared with 1·5 ml. of N/1 HCl and replaced in the incubator. After about ten minutes white flocculi appear. The mixture is centrifuged at high speed and the deposit suspended in 4 ml. of N/saline. N/10 NaOH is added, drop by drop, until the pH of the suspension is 7·5 after which it is vigorously shaken until the suspension is again dissolved. 1 ml. of 1 per cent. formol saline is added, the solution filtered through sterile lint, ampouled off in 5 ml. quantities and heated at 56° C. for two hours. This antigen is used in the test-proper in a dilution of 1:30. Each batch of antigen must be titrated before being brought into use.

Haemolytic System. This is prepared as for the Wassermann reaction, making a final suspension of red cells of 3 per cent. and using 5 M.H.D. of haemolytic serum.

Complement. This is prepared as for the Wassermann reaction (No. 1 M.R.C.), pages 87 and 90.

Test-proper

Three tubes are used for each serum tested.

Tube No.	1	2	3 (control)
Patient's serum	1	1	1 volume
Antigen suspension	1	1	— "
Complement (5 M.H.D.)	—	1	— "
Complement (3 M.H.D.)	1	—	1 "

The tubes are shaken and placed in the 37° C. water-bath for one hour. Sensitized R.B.C.s are then added, one volume to each tube, and the tubes shaken and returned to the water-bath. Results are read as soon as the serum-control tubes show complete lysis.

Reading results

" Strongly Positive " is indicated by—No lysis in Nos. 1 and 2 tubes.

" Positive " is indicated by—Lysis in first tube only.

" Negative " is indicated by—Complete lysis in all three tubes.

Coagulase Test for Staphylococci

This test is based upon the fact that certain strains of staphylococci, pathogenic to man and animals, are capable of coagulating citrated blood-plasma when incubated in its presence. A series of three Wassermann tubes is required for the test:

Tube No 1. (Organism to be tested.)

1:10 dilution of citrated plasma in N/saline	0·5 ml.
12-hour broth culture of the organism to be tested	3 drops

Tube No 2 (Control organism.)

1:10 dilution of citrated plasma in N/saline	0·5 ml.
12-hour broth culture of a known organism	3 drops

Tube No. 3. (Control plasma.)

1.10 dilution of citrated plasma in N/saline	0·5 ml.
Plain broth	3 drops

A " Positive " reaction shows coagulation of the plasma in Tube No. 1 within a few hours at 37° C. The control organism must also show coagulation whilst the control plasma must remain perfectly fluid

Agglutination of Staphylococci with Prothrombin-Fibrinogen Solution

Preparation of the Solution. To 30 ml. of oxalated human plasma, 10 ml of saturated solution of ammonium sulphate is added, mixed well and stood in the refrigerator for one hour. It is then centrifuged, the supernatant fluid decanted off and the tube filled with ¼-saturated ammonium sulphate solution.

The tube is again centrifuged, the supernatant fluid decanted off and the deposit dissolved in 20 ml. of 2 per cent. solution of sodium chloride containing 0·5 per cent. phenol. This is stored in the refrigerator. Any precipitate which forms from time to time may be centrifuged off. The stock solution keeps for at least three months.

The Test. One drop of the prothrombin-fibrinogen solution is placed on a clean slide and near it a small quantity of the bacterial growth (a single colony from a primary twenty-four-hour culture is suitable) is rubbed on the slide. Avoiding clumps of growth being carried over into the liquid, the growth is gradually mixed into the fluid. A "Positive" reaction, which appears almost immediately is indicated by marked granulation of the mixture whilst a homogeneous, milky appearance denotes a "Negative" reaction. The slide should be viewed against a dark background.

The Paul-Bunnel Test

(for Infective Mononucleosis-Glandular Fever)

This test is included here although it is questionable whether it is essentially "Immunological" technique. The test is based upon the fact that the blood serum of persons suffering from, or recently recovered from glandular fever will agglutinate sheep's red-blood corpuscles.

To perform the test a row of twelve Wassermann tubes are set up in a rack and into each tube is measured 0·5 ml. of N/saline. Into the first tube of the series 0·5 ml. of the patient's serum is measured and mixed with the N/saline; 0·5 ml. of the mixture is transferred to the second tube. This, in turn, is mixed and 0·5 ml. of the mixture transferred to the third tube and the same procedure carried out until the last tube is reached when the final 0·5 ml. of mixture is discarded. 0·5 ml. of a 2 per cent. suspension of washed sheep's R.B.C.s is added to each tube, followed by 1·0 ml. of N/saline. The tubes are shaken, placed in the 37° C. water-bath for one hour and then transferred to the refrigerator for forty-eight hours. Agglutination of the cells within the first five tubes indicates a "Positive" reaction.

Standardization of Vaccines

The approximate number of organisms per cubic centimeter of a vaccine may be estimated either by the use of the haemocytometer or by comparison with standard opacity tubes.

Haemocytometer method. The ordinary "Thoma" or "Neubauer" rulings are suitable for this purpose. The area of the squared part of the haemocytometer cell, together with the depth of the cell (between the surface of the slide and the under-surface of the cover-slip) are given on the slide. By making a known dilution of the suspension of organisms and counting the numbers of organisms present in a given number of squares the total approximate number of organisms in the original suspension may be calculated.

Example. Make a 1:20 dilution of the suspension of organisms by adding 0·1 ml. to 1·8 ml. of 1 per cent. formol saline plus 0·1 ml. of centrifuged Loffler's methylene blue. Place a drop of this dilution in the chamber of the slide (taking great care that it is not over-filled), allow it to stand for several minutes for the organisms to settle. Count the number of organisms in 80 small squares:

(*a*) The area of the squared portion of the chamber is 1 square millimetre (divided into 400 small squares).
(*b*) The depth between the surface of the slide and the cover-slip is $\frac{1}{10}$ millimetre.
(*c*) The dilution made is 1:20.
(*d*) We have counted 80 small squares which is $\frac{1}{5}$th of the total number (400).
So that the calculation is:
Number of organisms counted × 5 × dilution (20) × 10 × 1,000 (to give the number present in 1 c.cm.).
i.e. say 830 organisms were counted in 80 small squares.
Then 830 × 5 × 20 × 10 × 1,000 = 830,000,000 per c.cm. of the original suspension.

Opacity tube method (using Brown's standard opacity tubes)

The standard opacity tubes are obtainable commercially together with a table of equivalents for various organisms. Dilutions of the original suspension are compared with the tubes and the equivalent figure read off on the table. The result is multiplied by the dilution figure to obtain the number of organisms present in the original suspension.

CHAPTER XIV

GENERAL CULTURE TECHNIQUE

THE basic principle underlying all culture technique is the avoidance of contaminating microbes entering specimens and cultures during the short time that they are open at the bench.

Working Tools

Other than the various culture media in their respective containers the tools employed are simple, few in number and easily made.

Platinum loops and wires

Perhaps the most frequently-used pieces of apparatus in the Bacteriological laboratory are the platinum loop and wire (or needle). As we all know, platinum is an extremely precious metal and the greatest possible care must be exercised when using it. In view of its toughness and resistance to repeated heating it is ideally suited for this work and the expense involved is well justified. The life of loops and needles can be considerably prolonged and their scope enhanced if a few simple precautions are taken when making them. Having fixed a 2-in. length of platinum wire into a holder or embedded it in the end of a length of glass rod (see Fig. 18, page 109) make one complete turn of the wire round a match-stalk forming one coil of a spiral This will act as a " shock absorber " at this point, preventing the wire from breaking off short as so frequently happens at the most inopportune moments. Next, with a pair of fine-pointed forceps, a " square " loop is made at the tip of one wire providing not only a loop for transferring droplets of fluid, but sharp points of the square for touching colonies without fear of scratching the surface of media or spreading them unduly. Another difficulty experienced, particularly when dealing with slope cultures, is the possibility of fouling the surface of the medium with the end (or shoulder)

of the holder or rod. By making a double obtuse bend in the wire (see figure) this can be avoided. To make a platinum needle, proceed as for the loop except that the end is tapered by pinching it between pieces of fine ("0" gauge) emery paper and twisting it until the end is pointed. A slight curve in the wire close to the point will enable the worker to use the needle as a spreader when transferring colonies from one medium to another, without fear of scratching the surface of the medium. A platinum needle for making stab-cultures should be perfectly straight and of slightly thicker wire than that used for the loop Fine Nichrome wire (as used in electrical heating elements) may be used as a substitute for platinum.

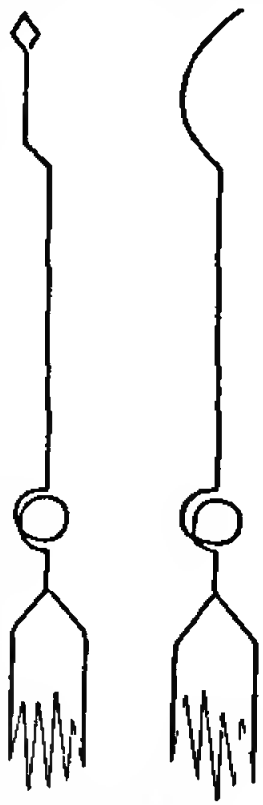


Fig 18
Platinum loop and needle

Glass Spreaders for distributing material over the surface of plates can be made from ordinary capillary pipettes and, if a series of half a dozen or so are kept in a rack they can be used in rotation many times over before being discarded (remembering, of course, that they must be heated to just below melting point of the glass after use). With the aid of the pilot flame of the bunsen burner soften the tip of a capillary pipette and allow it to droop over into a well-rounded curve With the tip-end of this curve held uppermost, heat the capillary until the curve begins to droop backwards. The finished spreader should be rather like an ordinary button-hook. Some workers prefer Drigalski's Spatula for spreading This consists of a length of 3 to 5 mm. glass rod with a double right-angle bend at one end.

Agar-gouge

It is sometimes desirable to divide the medium in a petri-dish into segments (see "Technique for Throat-swabs", page 217). A small strip of thin brass sheet, approximately 15 cm. by 1 cm. is folded in half along its length, leaving a channel approximately 2 mm. wide. One end is fixed into a wooden

handle and the other slightly bevelled and sharpened as shown in Fig. 19.

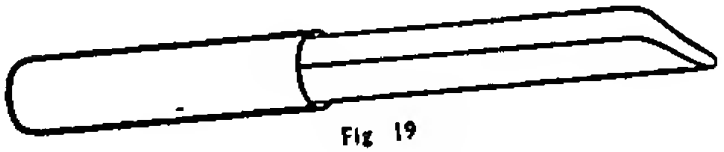


Fig 19
Agar-gouge.

Inoculation hood

For special culture techniques, where it is imperative that all extraneous organisms must be avoided, an inoculation hood may be employed. It consists of a frame with a slanting glass panel, a chimney, side-door for inserting a bunsen burner and two "cuffed" apertures in the front through which the operator passes his hands. The accompanying figure (Fig. 20) is self-explanatory and leaves little further to be said here.

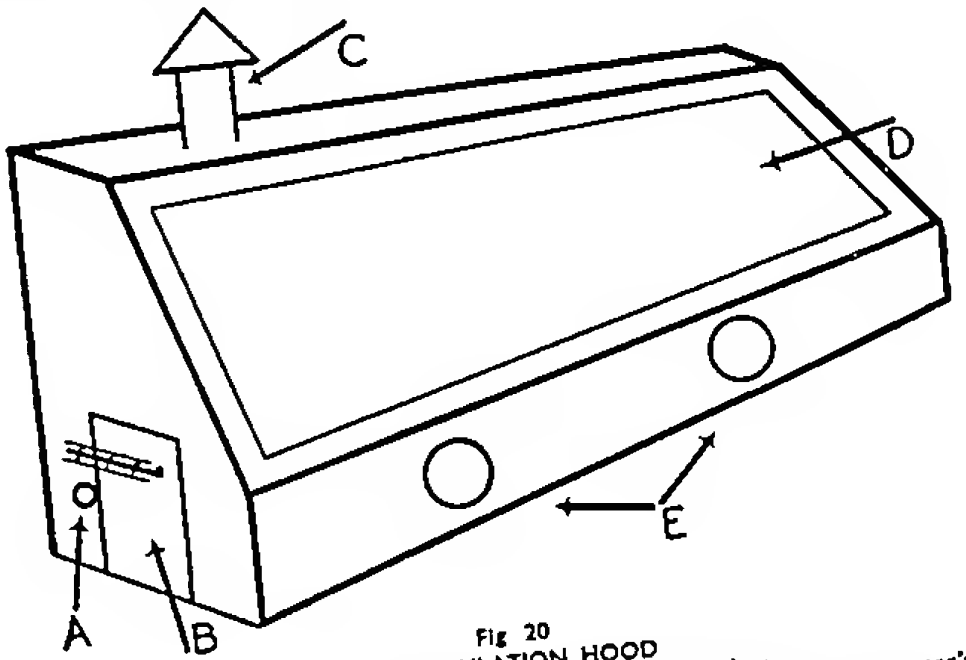


Fig 20
INOCULATION HOOD

A Hole for Bunsen tubing
B Trap door
C Bunsen chimney
D Glass window
E Gauntletted holes for operator's hands and arms

Incubators

The modern types of incubators are fitted with enclosed thermo-regulators, dispensing with most of the exposed movable parts common to the older models. Where the older types, comprising a weighted lever-bar and push-rod leading down to the capsule inside the apparatus, are in use,

efficiency can be maintained by attention to the following points:

(*a*) A drop of typewriter oil, placed on the upper part of the push-rod from the capsule, will run down the rod and prevent it from corroding to the side of the tube in which it moves.

(*b*) A soft cloth, lightly impregnated with vaseline, should be rubbed over the entire inner surface and all exposed metal parts of the incubator followed by wiping with a dry duster, about once a month. This will prevent corrosion through condensation of moisture from cultures.

(*c*) The contact-points of the regulator of electrically-heated models should be inspected regularly and kept free from dust. They may be cleaned by doubling a small strip of "00" emery paper and passing it between the points; no pressure other than that of the weighted lever must be applied for this, and the current must, of course, be switched off whilst it is done.

(*d*) Modern incubators fitted with bimetallic-strip thermostats of the "Sunvic" and similar types need little attention as the regulating device, including the make-and-break contacts are housed in dustproof covers.

An important point to bear in mind regarding many modern incubators and other electrically-heated apparatus is that the "pilot-light" (indicator-lamp) may indicate that the heaters are "ON" or "OFF" according to the models in use. A small card stating which of these apply should be fixed near the light.

Most makers of incubators and similar equipment include an "Instructions" card with each piece of apparatus and this card should be filed carefully and a typed copy either pasted on the inside of the door of the apparatus or hung near it.

Culture Technique

The term "culture", when used in the Bacteriological laboratory means a medium upon or in which living bacteria are growing. There are various types of cultures and these are referred to according to the type of preparation involved. A "Fluid culture" indicates organisms growing in a liquid medium such as peptone-water, broth, milk, etc., and such cultures are usually referred to according to the medium in

close together, across this line commencing at the wide end of the segment.

Labelling plate-cultures

Always label the bottom of the petri-dish (the half containing the medium). The risk of transposing lids is very real when comparing plates and could easily lead to very serious consequences. Practice labelling backwards so that writing can be seen "right-way round" through the medium (see Fig. 22b).

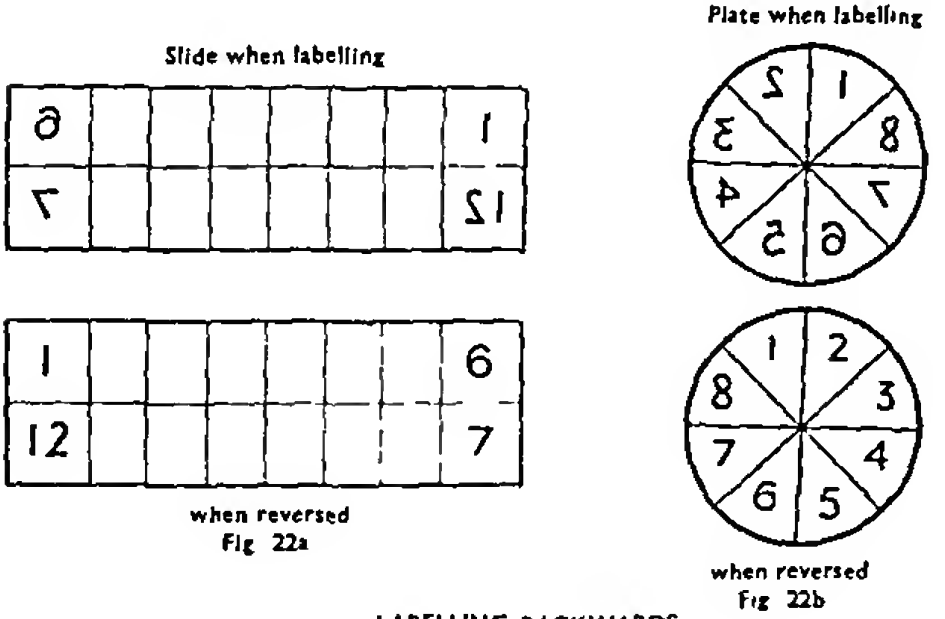


Fig 22a

Fig 22b

LABELLING BACKWARDS

Slope-cultures

Sufficient molten medium is poured into test-tubes or small screw-cap bottles to give a wedge of solidified medium that will provide a good surface upon which to grow organisms when the tubes or bottles are tilted on to their sides whilst the medium is cooling. Sufficient medium should be used to cover the entire bottom of the tube when it is sloped.

Stab-cultures

Molten medium is poured into test-tubes or small screw-cap bottles to a depth of approximately two-thirds the capacity of the vessel, sterilized and allowed to solidify. The organisms are implanted by stabbing an inoculated platinum wire into the medium, taking care that the medium is not split in the process.

Shake-cultures

These are prepared by melting tubes of medium and cooling to 45° to 50° C., inoculating the tubes with the organism and rotating the tubes between the palms of the hands to obtain an even distribution of the organisms throughout the medium and then, either allowing the medium to solidify or, if plate cultures are required, pouring the inoculated medium into a petri-dish and allowing it to solidify.

Isolation of Organisms from Mixed Cultures

A " pure " culture is one which contains only one particular type of organism and pure cultures may be obtained by either (*a*) *Plating*, dilutions of the mixed culture are spread over the surface of plates, using one of the methods described on pages 112 and 113, and isolated colonies selected and transplanted on to fresh medium, or (*b*) *Pour-plating*, the mixture of organisms is diluted and added to molten medium (cooled to 50° C.), mixed thoroughly with the medium and plates poured (as for shake-cultures above) Series of dilutions may be obtained by transferring loopfuls of inoculated medium into further tubes of molten medium and pouring plates from the series of dilutions thus obtained.

Note.—When " burning off " infected loops or needles; the instrument should not be plunged directly into the flame as this will frequently cause the material on the instrument to " sputter " and droplets will be spread over the bench. The heating should be done gradually, starting with drying the material close to the flame.

CHAPTER XV

ANAEROBIC CULTURE TECHNIQUE

IT is not so much the PRESENCE of oxygen that is against the growth of anaerobic bacteria as the formation by them of hydrogen peroxide during growth (or attempt at growth) in the presence of free oxygen. This can be demonstrated by the addition to culture media of a catalyst such as small pieces of fresh tissue, as in Smith-Noguchi's medium or by the replacement of the air surrounding the medium by an inert gas or by the total exclusion of air.

If we take a tube of fluid medium and add a small piece of fresh sterile rabbit kidney the lower portion of the medium in the tube containing the kidney will support growth of known anaerobic bacteria because the kidney will break down the hydrogen peroxide as rapidly as the bacteria produce it.[1] Again, if we drive off all the air from a tube of medium and prevent any further air reaching the medium, or replace the air in the tube with hydrogen or carbon dioxide the medium will support growth of anaerobic bacteria. Further, if we place actively aerobic bacteria in close proximity to anaerobic bacteria and limit the air supply to that already surrounding both cultures, both will grow.

Various methods for maintaining anaerobiosis have been devised, and one or more of the following may be found in regular use in most Bacteriological laboratories.

Method 1. The surface of broth (or whatever fluid or semi-fluid medium may be chosen) in tubes is covered with a layer of liquid paraffin and the tubes autoclaved. The dissolved air is thus driven off through the layer of liquid paraffin, but no air can re-enter the medium through it. The tubes are inoculated with the material under investigation with a capillary pipette passed into the medium through the paraffin. This method may be applied to most liquid media and to semi-solid agar. With semi-solid agar only partial re-absorption of air occurs following heating, if the paraffin is omitted, and some growth

[1] See Chapter XVII for Bacterial enzymes.

will occur in the lower portion of the tube only. Brewer adds 0·1 per cent. sodium thioglycollate to semi-solid agar to maintain anaerobic conditions, dispensing with the paraffin layer (see "Culture Media", page 167)

Method 2. (Removal of free oxygen by another organism.) Two plates of solid medium are poured, in dishes of the same

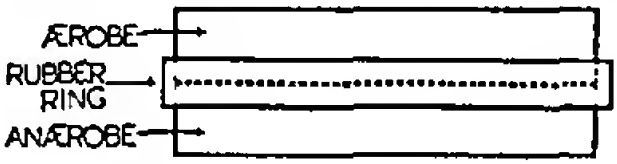


Fig 23

diameter (and with undamaged edges). One plate is inoculated with an actively aerobic organism such as pseudomonas aeruginosa, whilst the other is inoculated with the anaerobe. The two lower halves of the petri-dishes are placed together and sealed with a stout, tight-fitting rubber band (see Fig. 23).

Method 3. Buchner's tubes. A mixture of pyrogallic acid and caustic soda will absorb large quantities of oxygen. By

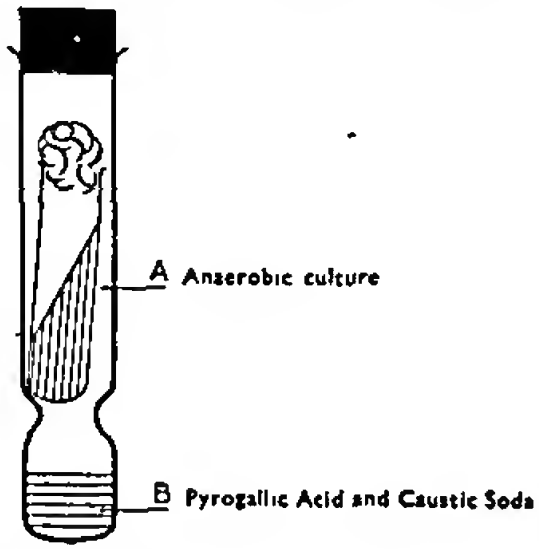
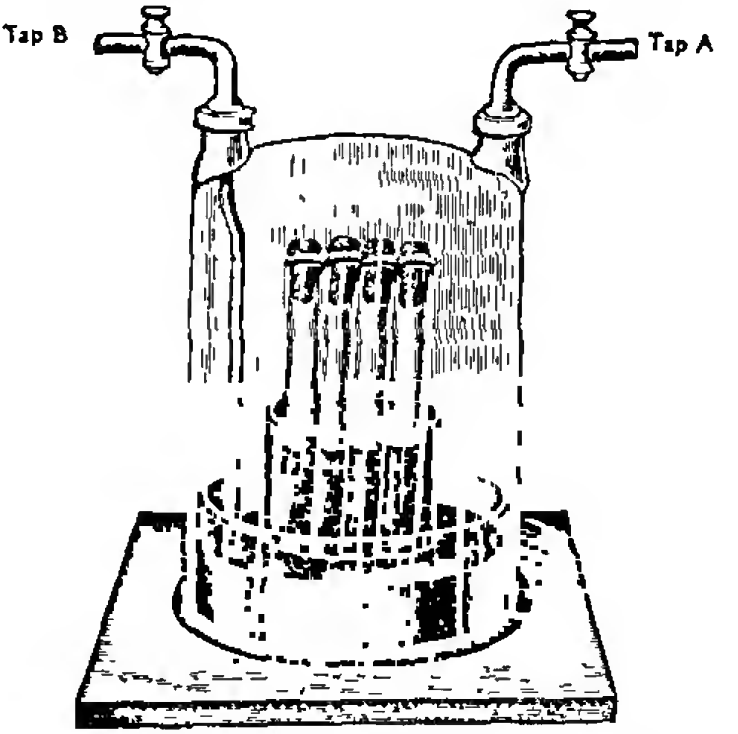


Fig 24
Buchner's tube

placing pyrogallic acid into the lower bulb of a Buchner's tube, inserting the culture tube, quickly running a small quantity of 40 per cent. NaOH down the inside of the Buchner tube and then replacing the rubber bung, the oxygen present inside both tubes is absorbed, leaving only nitrogen (an inert gas) present (Fig. 24).

Method 4. **Bulloch's Jar.** This apparatus allows for a number of tubes or plates to be cultivated at the same time in the one apparatus. The cultures are placed in the inner glass dish which is stood in the centre of the ground-glass plate. The cover is replaced and sealed on to the ground-glass plate with a thin coating of petroleum jelly. Both taps are opened and hydrogen is passed into tap " A " and gass escaping from tap " B " is tested by placing a test-tube over it for a few



(By courtesy of Messrs A. Gallenkamp)

Fig 25
Bulloch's Jar

moments and, closing the test-tube with the thumb conveying the tube to a bunsen flame and releasing the thumb with the mouth of the tube close to the flame. A mixture of hydrogen and air explodes whilst pure hydrogen burns with a steady blue flame. The latter indicates that practically all the air inside the apparatus has been replaced by hydrogen. It is important to remember that hydrogen is highly inflammable and no naked flames must be near the generator, hence the advice to CONVEY THE TEST-TUBE TO A FLAME. When the contents of the tube burn with a steady blue flame at the rim of the tube, both taps are closed and the apparatus transferred

to the incubator. The illustration (Fig. 25) is of a type of apparatus which includes a shallow dish inside the jar for containing pyrogallic-soda mixture to absorb any remaining traces of oxygen.

Method 5. McIntosh and Filde's Jar. This is a highly efficient apparatus but involves more preparation than most other methods. The apparatus consists of a large thick-walled glass jar fitted with a metal lid through which pass two taps and two terminals. The terminals are connected on the inner side of the lid to a wire filament passing through palladinized asbestos contained in a wire gauze cage. To operate, the jar is opened, the cultures inserted and the lid replaced and clamped tightly. One tap is opened, connected to the vacuum pump and the apparatus exhausted. It is then closed. The other tap is connected to the hydrogen generator and hydrogen allowed to enter the apparatus. These two processes may be repeated for greater efficiency. After the final filling with hydrogen both taps are closed and an electric current passed through the filament to heat the palladinized asbestos. This will cause the combination of any remaining traces of oxygen with hydrogen to form water vapour which will condense on the inner surface of the metal lid The current is switched off, the wires disconnected and the apparatus transferred to the incubator. The amount of current required will vary with the thickness of the resistance wire used to heat the palladinized asbestos, but in most cases the current passed by one 40-watt lamp, using ordinary domestic supply of electricity, is sufficient.

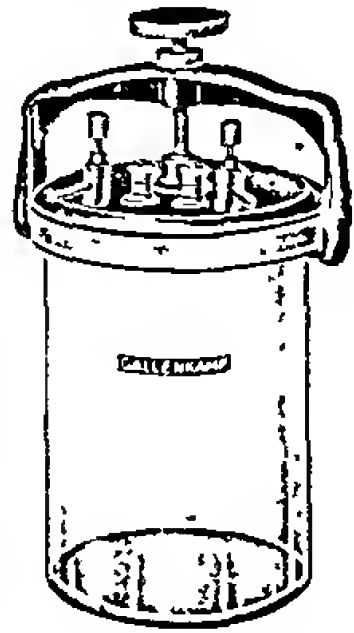


Fig 26
McIntosh and Filde's Jar

The supply of hydrogen may be obtained by using an ordinary Kipp's apparatus containing granulated zinc and dilute sulphuric acid or a "MICROID" gas generator (an extremely efficient apparatus supplied by Messrs. Griffin and Tatlock). The gas should be passed through three wash-bottles containing 10 per cent. lead acetate, 10 per cent. silver nitrate and a mixture

of pyrogallic acid and caustic soda respectively to remove impurities before the gas passes into the anaerobe apparatus.

Method 6. Home-made " Anaerobe tins " which work very well can be made in the laboratory from old golden syrup tins (or similar).

Two small angle tubes are soldered into the side of a tin and a short length of glass tubing fitted with an " indicator " fixed between them by means of short lengths of rubber tubing; the upper piece of rubber tubing is fitted with a screw-clip (Fig. 27). Cultures are inserted into the tin and the lid sealed

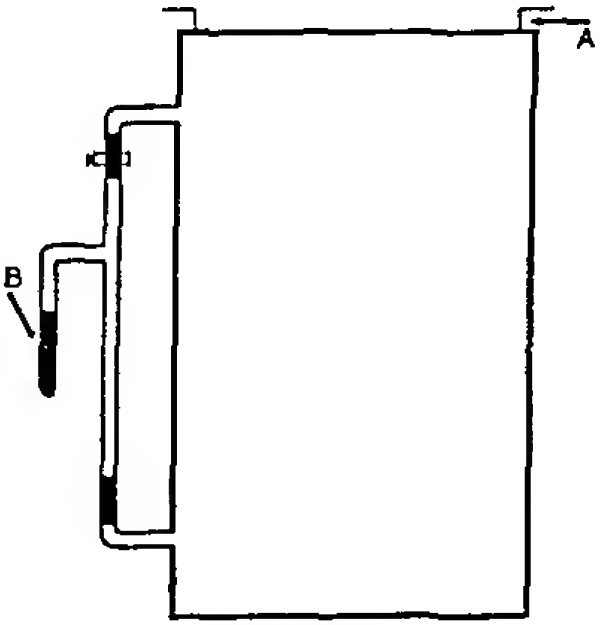


Fig. 27
Home-made Anaerobe tin
A—Lid B—Indicator-tube

with plasticine. The upper angle tube is attached to the hydrogen generator. Escape of pure hydrogen is tested for at the lower angle tube as in Method 4. When the apparatus is filled with hydrogen the screw-clip is closed, the glass " indicator " tube inserted between the rubber connections and the clip opened again. If true anaerobiosis has been achieved the indicator will gradually lose its colour.

A solution of 0·01 per cent. methylene blue in 1 per cent. glucose may be used as an indicator.

CHAPTER XVI

STOCK CULTURES

WHERE stock cultures are frequently in use, such as in training establishments, most bacteria may be maintained on plain nutrient agar slopes; sub-cultivation on to fresh slopes being made at intervals according to the type of organisms involved

Where parasitic and semi-parasitic bacteria are concerned, the addition to the medium of blood, serum, ascitic fluid or other body-fluids is necessary and sub-cultivation must be done more frequently, in some instances even daily.

Two main causes for the "dying out" of cultures exist. Firstly, the accumulation of waste products of metabolism of the bacteria in the surrounding medium and, secondly, slow drying. Nutrient agar is preferable to broth as the soluble waste products of the bacteria tend to diffuse away from the growing colonies into the substance of the medium, whilst in broth the bacteria are suspended in a solution of their waste-products. Frequent sub-cultivation overcomes the problem of auto-intoxication of bacterial cultures, but slow drying of cultures that would otherwise remain viable for long periods can be dealt with by one of the following methods:

(*a*) Use of screw-cap bottles (see page 143).

(*b*) Impregnating cotton-wool plugs with paraffin wax.

(*c*) Sealing off the tops of tubes in the blow-pipe flame.

(*d*) Covering the surface of the medium, after inoculation and a short period of incubation, with sterile liquid paraffin or other mineral oil.

Whilst method (*a*) has the advantage of simplicity and comparative safety from contamination it does not prevent, or retard, dissociation of bacteria during storage as does method (*d*). The drawback of method (*b*) is that subsequent upon sealing a certain amount of moisture is condensed on the cotton-wool plug inside the tube which is sufficient to promote germination of any possible mould spores present in the wool followed by the development of hyphae which will gradually

grow down the sides of the tube to contaminate the medium. Method (*c*) has many disadvantages, not the least of which is the grave danger to the worker when opening the sealed tubes.

Many bacteria may be preserved for indefinite periods of time by rapid dessication in the cold. Cultures are first frozen, either in the refrigerator or with "dry ice" (solid CO_2) and then placed in a vacuum desiccator over sulphuric acid and the desiccator evacuated. It is claimed that bacterial cultures so treated remain viable, and morphological, biochemical and antigenic characteristics remain unchanged almost indefinitely.

CHAPTER XVII

PREPARATION OF CULTURE MEDIA

BEFORE we proceed with the subject of culture media and its preparation, it would be well for us to briefly consider enzymes and their action generally—particularly as the value of many media lies in their ability to demonstrate specific enzymic action of micro-organisms. A knowledge of bacterial enzymes gives us a better appreciation of the important roles which various ingredients (apparently superfluous at times !) may play.

Function of Enzymes

In chemistry we are taught that a CATALYST is any substance which can take part in a chemical reaction without, itself, being used-up, altered in character or taken-up as part of any new substance formed by the reaction. Having played its part the catalyst can again be equally active in the presence of further quantities of the original substances.

E.g. :

(*a*) " A " plus " B " plus CATALYST gives " AB " plus CATALYST and if we now transfer the catalyst to further quantities of " A " and " B " we again obtain " AB " plus catalyst and so on

(*b*) " XY " plus CATALYST gives us " X " plus " Y " plus CATALYST and upon transferring the catalyst to a further quantity of " XY " we again obtain " X " plus " Y " plus catalyst and so on.

Enzymes function in a similar manner to catalysts and for this reason they are frequently referred to as BIOCATALYSTS because they are produced by or contained within living things.

Composition of enzymes

It is generally accepted that enzymes are of very complex chemical structure, the nature of which is, in most cases still a mystery. A few enzymes have been prepared in pure crystalline form by expert chemists but detailed knowledge of these

scarcely comes within the province of the medical laboratory technician. It is best, therefore, for us to accept the fact of their existence and infinite variety by the evidence of their action and the selectivity they may exhibit in their action upon certain substances.

E.g. :

An enzyme which acts upon sugars may have no effect whatsoever upon proteins.

(*Note.*—When discussing in general terms, substances upon which enzymes act, we use the word SUBSTRATE to describe these substances.)

Conditions affecting enzymic action

Two main factors influence the activities of enzymes : TEMPERATURE and REACTION (pH or Hydrogen ion concentration) of the substrate. With regard to temperature, most enzymes have an OPTIMUM (most suitable) temperature at which their activity is at its peak. Temperatures below this slow down activity more or less proportionately, but freezing does not kill (INACTIVATE) them. Higher temperatures have similar effects up to a point where the enzyme is completely inactivated (destroyed). This point varies for different enzymes.

Reaction of the substrate plays an important role as some enzymes will function only in an acid medium whilst others may prefer an alkaline one. Yet other enzymes prefer neither acid nor alkali (neutral).

Occurrence of enzymes

All living things possess enzymes of which there seems to be an almost infinite number. Micro-organisms are no exception to this. Some enzymes have a specific action upon one particular substance only ; others may act upon a number of substances and, yet others, will break down highly complex substances through a series of stages, forming distinct recognizable substances at each stage.

Many micro-organisms appear to possess a number of separate enzymes (or it may be that they possess single enzymes which are capable of action upon a variety of substances—it

is not yet clear which !). The enzymes of micro-organisms may be broadly divided into two main types :

(*a*) EXO-enzymes, which are excreted by the living cells and remain in solution in the substrate. If, subsequently, the organisms are removed the liberated enzymes will remain behind in solution and will continue to be active

(*b*) ENDO-enzymes, which remain within the living cells. If organisms are removed intact from the substrate no further enzymic activity takes place Such enzymes may, however, be released into the substrate if the organisms are damaged or disintegrate

Naming enzymes

The naming of enzymes has been somewhat simplified—in so far as so complex a subject can be simplified !—by the use of the suffix " -ase " as the last syllable of the name of the substance upon which a particular enzyme or group of enzymes acts.

E.g. :
The enzymes which break down proteins are known as PROTEASES, whilst those which break down carbohydrates (sugars) are known as CARBOHYDRASES. The enzyme which breaks down cellulose is CELLULASE and so on.

Some enzymes are presumed to be, in reality, mixtures of enzymes because of their ability to break down a variety or a mixture of substances (as in gastric juice). Where these occur the suffix " -in " is added as in PEPSIN which is present in the intestine of humans and some animals.

Classification of enzymes

As can well be imagined, with the multitudinous activities of enzymes, coupled with the still far from complete understanding of their structure, classification becomes a very complicated and—no doubt—somewhat inaccurate business. For our purpose we may broadly classify enzymes of micro-organisms into two groups : HYDROLASES and DESMOLASES.

Carbohydrases include many enzymes of which the following are but a small representative number :

ZYMASE—reduces glucose and fructose to alcohol and CO_2.
MALTASE—reduces maltose to glucose and fructose:
SUCRASE (Invertase)—reduces sucrose to glucose and fructose.
LACTASE—reduces lactose to glucose and galactose.
AMYLASE—reduces starch to maltose and dextrin.

Proteases

These enzymes are responsible for breaking down proteins through various stages to simple amino-acids. They include PROTEINASES and PEPTIDASES. Proteinases break down proteins to peptones and polypeptides whilst the peptidases carry the decomposition further, reducing these to amino-acids.

Lipases

These enzymes reduce fats to fatty acids and glycerol.

E.g. :
Bacterial action in butter may produce a condition we all have met—RANCIDITY.

Phosphatase

This enzyme is produced by yeasts and is also present in milk. The official " PHOSPHATASE TEST " for heat-treated milk is based upon the fact that this enzyme is inactivated at a temperature of 63° C. (145° F.). If it is claimed that a milk has been pasteurized and the milk, when tested is found to contain phosphatase then the process of pasteurization has not been carried out efficiently.

Oxidases and dehydrogenases

These enzymes are said to be the " RESPIRATION " and " FERMENTATION " enzymes because of their ability to " split off " atoms of hydrogen from one substance and attach them to other substances. When the " split off " hydrogen is attached to free oxygen the process is said to be one of respiration but when these atoms are attached to a substance other than oxygen it is one of fermentation. Oxidases will only

Cultivation of micro-organisms

Direct microscopical examination of specimens for the presence or absence of micro-organisms is not, in itself, sufficient, except perhaps in certain specific instances such as the routine examination of sputa for M. tuberculosis, blood films for malarial parasites, or fresh stools for amoebae, etc.

In order, therefore, that a study can be made of the many characteristics by which bacteria are identified and classified, cultures must be obtained on artificially prepared foods (media). Whilst some micro-organisms are able to exist and multiply under a wide range of conditions, other more exacting species are very selective in their requirements regarding food, temperature and the reaction of the medium in which they are to grow.

Unlike most small boys, bacteria cannot absorb vast quantities of indigestible foods! They do not possess digestive systems as do the higher forms of animal life and it is necessary for foods to be broken down (pre-digested) into simple substances which the bacteria are then able to absorb.

Some organisms, viruses for example, will only survive and multiply in living tissues and special means for cultivation have to be employed. Others will flourish only if certain body fluids (serum, blood, ascitic fluid, etc.) are added to the medium.

An interesting feature in the cultivation of bacteria on artificial media is the fact that an organism originally requiring serum or blood can sometimes be " trained " to exist without these substances, eventually growing profusely upon quite simple media.

Repeated passage of the organism through a series of living hosts will cause such organisms to revert to their original habits, losing this acquired ability to utilize simple media.

As bacteria become more and more dependent upon a living host (parasitic), not only do they lose their enzymic properties (power to break down complex compounds to simple substances), but they decrease in substance, again as evidence the viruses which, as far as is known at present, depend entirely upon the enzymes of their hosts and which are ultra-microscopic in size.

Bacteria which are capable of "foraging for themselves", such as the Clostridia for example, possess enzymes enabling them to break down complex organic matter into the simple substances which they require as food. Such bacteria are known as Saprophytes.

Various species of bacteria are able to break down (ferment) carbohydrates (sugars) and many bacteria are selective in their choice of sugars This selectivity can be put to good purpose in the laboratory as part of the system of identification of species. Some of the sugars are destroyed or converted into totally different substances by excessive heating and this fact must be borne carefully in mind when choosing methods for sterilizing media containing them.

Moisture is essential to bacterial growth and, except in certain circumstances such as rapid drying *in vacuo* or in the case of the spore bearers, drying kills bacteria. With regard to the spore bearing bacteria, these are able to withstand many adverse conditions in addition to drying, such as comparatively high temperatures and exposure to disinfectants for varying lengths of time; hence the need for special technique for sterilizing ("Fractional Sterilization", see page 68).

Some species of bacteria are able to break down inorganic chemicals and utilize them as food. This, again, can be employed in the laboratory as a means of differentiating between species which may otherwise resemble each other.

Most bacteria prefer a slightly alkaline medium (in some instances the presence of even minute quantities of acid may inhibit or retard growth). A few species prefer an acid medium, but the majority of bacteria will thrive in a medium which is practically neutral in reaction. As slight excess of acidity or alkalinity may adversely affect cultures, it is necessary for the reaction to be accurately adjusted. .

Peptone

This is a loose term used in the laboratory for a commercial product containing a number of simple organic substances (proteoses, peptones, amino-acids, etc.) which bacteria are able to utilize as food.

Meat extracts

These provide certain mineral salts, sugars and organic nitrogenous substances in balanced proportions suitable to the growth of many bacteria.

Mineral salts

Minute quantities of inorganic chemicals such as chlorides, phosphates and sulphates among the acid radicles and sodium, potassium and calcium among the metallic radicles, are included in culture media either forming part of the meat extract or as additions during preparation.

Certain dyes and chemicals, in varying dilutions, exert an inhibitory effect upon some bacteria, but not upon others. This fact can be utilized in the preparation of media required for isolation of specific organisms from mixtures of bacteria, particularly where the species sought are likely to be "overgrown" by more hardy types.

It is safe to assume that most formulae and methods of preparation are subjected to exhaustive tests before being finally adopted for use. Each ingredient, each stage in preparation plays its part and has a definite bearing upon the final medium. It is very important that side-stepping, short-circuiting, substitution and omission of ingredients should be avoided when preparing culture media. It is far wiser to admit an error or doubt regarding one's technique and to "scrap the whole thing" and start afresh than to take chances in this branch of the work.

The essentials of good media-making are:

(*a*) Great care in measuring and weighing ingredients.
(*b*) Correct timing of processes involved.
(*c*) Accuracy in adjusting reactions.
(*d*) Strict avoidance of over-heating.
(*e*) Strict adherence to formulae and methods of preparation.

A number of basic solutions and stock digests form the bulk of routine laboratory media and these, together with special media in more or less regular demand are described in the following text.

Records of all media prepared should be kept in the media-room so that reference may be made to any particular batch of media. Batches of media should never be mixed and each batch should be clearly labelled.

Sterilization of media

The three most common methods for sterilizing media are: Autoclaving, Steaming and Inspissation. In certain circumstances Filtration is employed (see page 181).

Adjustment of reaction of media

The method of adjusting the reaction of media most commonly used is known as the pH or "Hydrogen Ion Concentration" method, although a simpler but less exact technique, "Eyre's Scale", may be used. A knowledge of the latter is an advantage against the possibility of damage or temporary loss of the special apparatus required for the former.

The pH method is based upon the principle that the acidity of any fluid depends upon the number of hydrogen ions present in the fluid. The reaction of the fluid depends upon, and can be accurately measured by determining, the concentration of hydrogen ions present in it. The hydrogen ion content is symbolized by the letters "pH" and the number given with these letters represents the degree of acidity or alkalinity of the fluid: pH 7 being neutral point. Various dyestuffs change colour over different pH ranges and can, therefore, be used as indicators according to the particular range required. For example:

Indicator	pH range	Colour change
Thymol blue (acid orange)	1·2–2 8	Red to orange.
Phenol red	6 8–8·4	Yellow to purple-pink
Cresol red	7·2–8·8	Yellow to violet-red.
Phenol phthalein	8·3–10·0	Colourless to red.

As the reaction required for most culture-media lies between pH 6·8 and 8·4 phenol red is most frequently used as the indicator when adjusting its reaction.

Method. Apparatus required:

One 5-ml. or 10-ml. delivery pipette.
One 1-ml. graduated pipette.
Two 10-ml. micro-burettes (graduated in $\frac{1}{10}^{ths}$ ml.).
One standard pH comparator set (phenol red range).

Reagents required:

N/10 Sodium hydroxide solution.
N/1 " " "
N/10 Hydrochloric acid solution.
N/1 " " "
Distilled water.
0·01 per cent. aqueous solution of phenol red.

The tubes of the comparator-set are placed in the partitions provided in the set as illustrated in the diagram (Fig 28).

(*a*) 5 ml. of medium are measured into tubes "A", "C" and "E".

(*b*) Distilled water is poured into tube "B".

(*c*) Buffer-tube pH 7·2 is placed in "D" and pH tube 7·6 in "F".

(*d*) 0·5 ml. of phenol red indicator is added to tube "E".

Let us assume that 1 litre of broth is to be adjusted to pH 7·4 and, when the indicator is added to tube "E" it is found to be acid. A burette is filled with N/10 sodium hydroxide solution and this is run into tube "E" a drop at a time until the colour, seen through "E-B" (through the small aperture in the side of the set) is midway between the colours of "A–D" and "C–F".

The amount of N/10 alkali used is noted.

E.g.:

The 5 ml. of medium in tube "E" required 0·61 ml. of N/10 alkali

re 61 ml.
ire 122 ml. of N/10 or 12·2 ml. of N/1 alkali.

But 15 ml. of the medium have been used for the test and 10 ml. of this may be returned to the bulk, leaving 5 ml. expended.

If 1,000 ml. required 12·2 ml. N/1 alkali

Then 995 ml. requires $\frac{12{\cdot}2 \times 995}{1{,}000} = 12{\cdot}139$ ml. of N/1 NaOH

The tube "E" is emptied, washed with distilled water and allowed to drain. 5 ml. of the adjusted medium is measured

into it, 0·5 ml. of indicator added and the tube replaced in the comparator set which is rearranged as follows:

5 ml. of medium is placed in tube "A"
Distilled water in tube "B"
pH 7 4 buffer-tube placed in "D".

The tints through "A–D" and "E–B" should now match; should this not be so, further adjustment with either acid or alkali must be carried out.

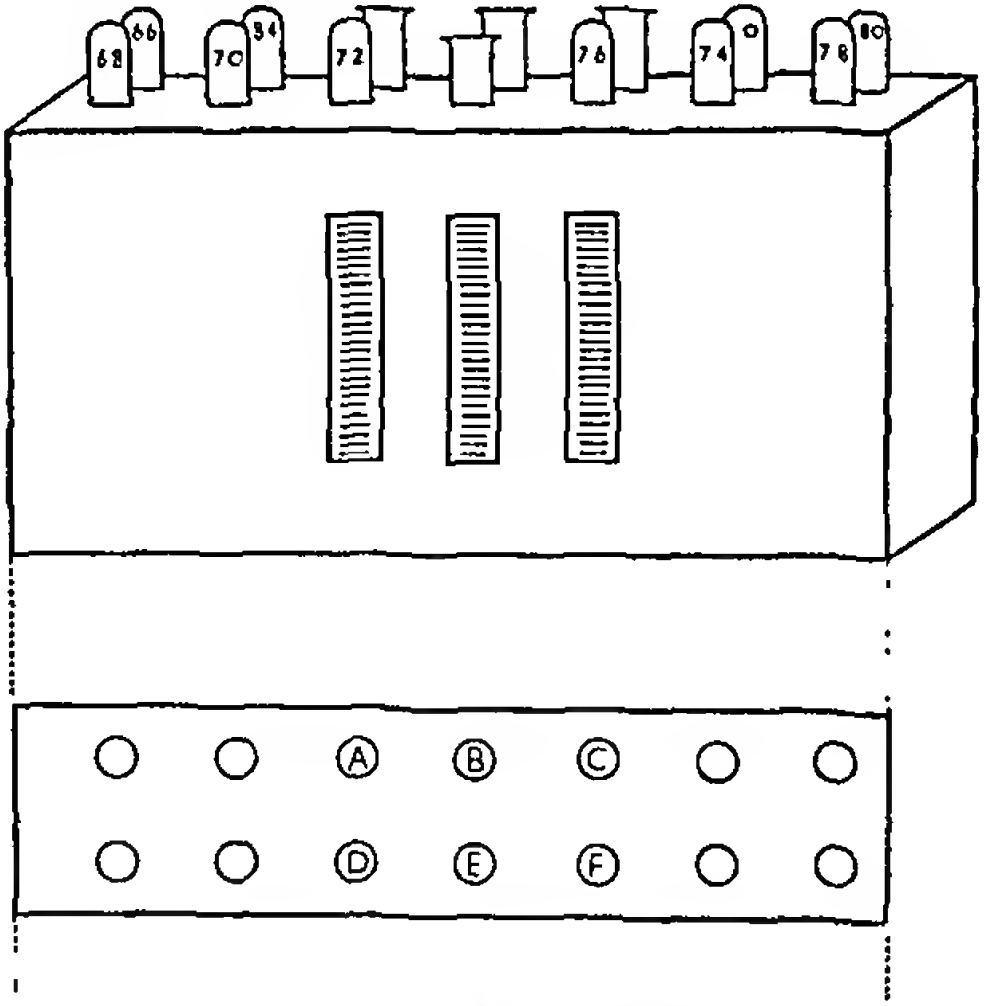


Fig 28

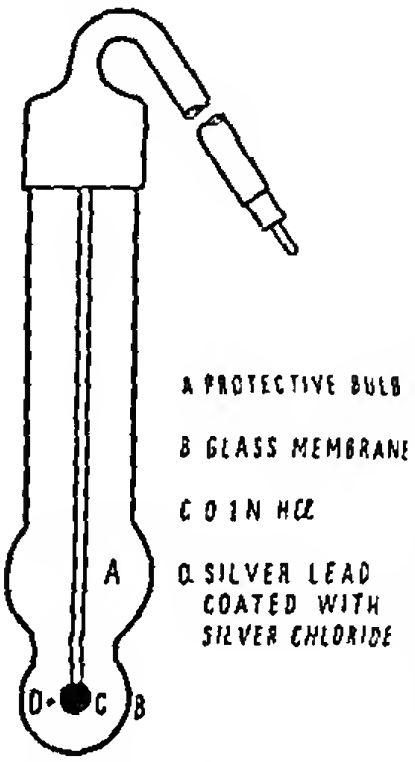
A. 5 ml medium
B Distilled water
C 5 ml medium
D Standard tube pH 7 2
E 5 ml medium + 0 5 ml 0 1 % phenol red
F Standard tube pH 7 6

Note.—There is a possibility of the standard buffer-solutions in this form of comparator fading if exposed to strong lights or to excessive heat, as may be encountered in tropical and sub-tropical climates. "LOVIBOND" disc comparators are an efficient substitute, and are used in a similar manner to the

above; merely inserting a disc of colours of the pH range required and using the appropriate indicator. The discs are notched and protrude just beyond the edge of the comparator enabling the user to revolve the disc at will to bring the required tinted glass into position for comparisons.

The Use of the Glass Electrode

The indicator method of ascertaining the pH of fluids suffers a number of disadvantages which in practice limit its application. The most obvious of these occurs in the case of coloured solutions where difficulty may be experienced in detecting changes in the colour of the indicator. Somewhat less obvious sources of error are produced by the presence of large concentrations of neutral salts and by adsorption effects of protein solutions. The two latter may cause errors up to 0·5 pH unit.



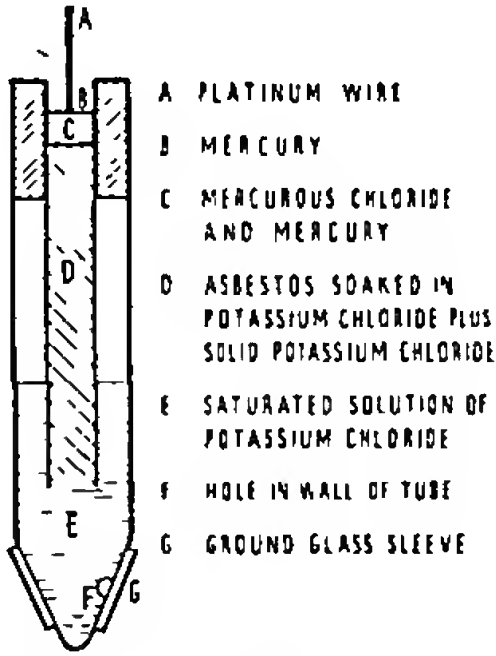
GLASS ELECTRODE
Fig 29

A number of so-called electrometric methods have been developed during the past decade or so which obviate some of the errors in the indicator method and, moreover, allow of the rapid and accurate adjustment of pH over a considerable range. The better known of these methods employ what is known as a *glass electrode.*

The glass electrode (Fig. 29) consists of a thin film of special glass in the form of a bulb which is sealed on to a stem. The bulb contains a solution of constant pH value, dipping into which is a suitable metal lead (for example: a solution of hydrochloric acid at pH 1 is commonly used with a silver electrode coated with silver chloride).

If this electrode is immersed in a solution having a pH differing from that inside the bulb, a potential develops across the glass film which is proportional to the difference in pH

between the two solutions. This potential is measured by inserting suitable electrodes into the two solutions and connecting these to a potentiometer. One of these electrodes is already provided in the glass electrode as described above, whilst that normally employed for insertion into the test fluid is a *calomel half-cell.* The half-cell consists of a mixture of mercurous chloride and metallic mercury, having a platinum wire as connecting lead, enclosed in a narrow tube as in Fig. 30.



CALOMEL ELECTRODE

Fig 30

Below this mixture, which constitutes the electrode proper, is a plug of asbestos and solid potassium chloride wetted with a saturated solution of the latter; this tube being immersed in a saturated solution of potassium chloride contained in an outer tube. Electrical contact with the test solution is made by means of a film of the potassium chloride solution held in the ground-glass sleeve. Other types of calomel half-cell are used, but differ only in design and not in principle.

Just as a potential is set up across the glass membrane on insertion into a solution so are potentials acquired by the two electrodes with the result that the potential difference or ELECTROMOTIVE FORCE (EMF) measured by the potentiometer

is the sum of the individual ones. However, at constant temperature the potential exhibited by the calomel electrode and silver/silver chloride are constant as also is the potential between the inside of the glass membrane and the solution of constant pH. Neglecting any potential set up between the test solution and the saturated potassium chloride solution, the only variable at constant temperature is, therefore, the potential at the outside of the glass membrane in contact with the solution. The state of affairs existing in the system may be depicted as follows, where E is the potential in each case. (Fig. 31.)

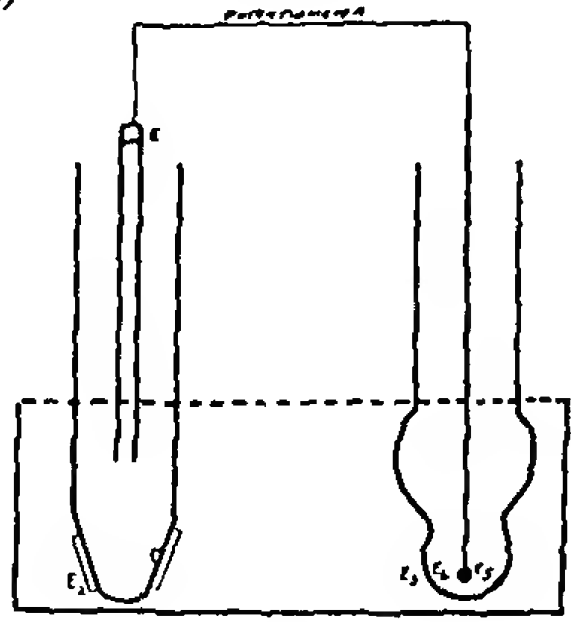


Fig 31

Total E equals $E_1 + E_2 + E_3 + E_4 + E_5$ equals Constant + E_5 at constant temperature.

If we denote changes in E by ΔE and in pH by ΔpH then at constant temperature

$$\Delta E = \Delta E_5 = K \Delta pH \text{ where } K \text{ is a constant.}$$

The measurement of ΔE by means of a potentiometer in the normal way (see Fig. 32A) is impossible owing to the very high resistance of the glass electrode system and to the fact that such a method requires that a steady current passes through the calomel-glass electrode system until a balance is obtained. This latter would result in the partial or complete discharge of the various potentials (i.e. in a change of EMF in the system, or as it is commonly called, Polarization).

The galvanometer in the potentiometer circuit may be

replaced by a single triode valve (see Fig. 32B), the EMF to be measured being applied to the grid of the valve. In this way a negligible current is drawn from the system whilst the current flowing in the anode circuit is a measure of the applied EMF.

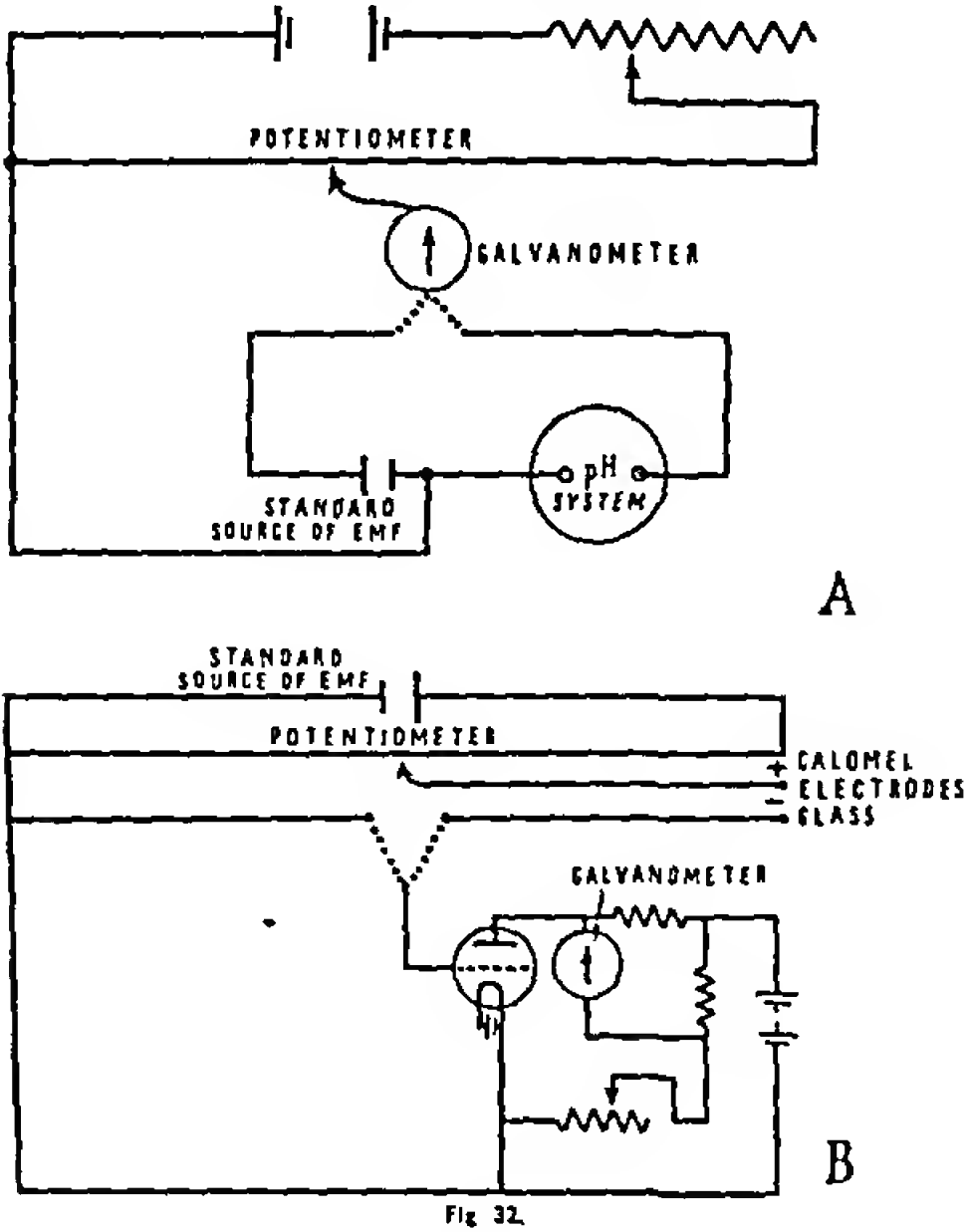


Fig 32.

The two methods of EMF measurement are compared in the diagrams (Fig. 32AB).

The measuring system involved in Fig. 32B is known as a valve-electrometer and modern pH recording and measuring instruments employ valve electrometers developed from this or similar circuits.

In practice the electrometer potentiometer is calibrated directly in pH units and the instrument is "SET" against a solution of known standard pH (usually a buffer solution) falling within the range over which measurements are to be made. Temperature compensation is achieved either by a hand-operated knob or, in many mains circuits automatically.

The majority of pH measuring instruments are supplied with a complete set of instructions as to their use and little would be gained in recounting them here. It may be noted, however, that ΔE may not be strictly proportional to ΔpH under conditions of extreme pH, and standardization against buffer solutions in the same pH range is essential.

pH papers

Small books of papers impregnated with various indicators for specific pH ranges are now procurable (B.D.H.) and are valuable additions to the media-room equipment for rapid rough checking of the reaction of media.

Eyre's Scale

Apparatus required:

Bunsen burner, tripod and gauze.
Two 6-in. evaporating dishes.
One 10-ml. pipette.
One glass stirring rod.
Two 10-ml. burettes (graduated in $\frac{1}{10}$ths ml.).

Reagents:

N/10 Sodium hydroxide solution.
N/1 „ „ „
N/10 Hydrochloric acid solution.
N/1 „ „ „
Distilled water.
Phenol phthalein solution (0·5 per cent. in 50 per cent. alcohol).

The test is carried out at boiling point.

The two evaporating dishes are labelled "A" and "B". Into each dish is measured 10 ml. of the medium to be adjusted and an equal quantity of distilled water added. (As most media

are coloured this dilution enables "end-points" in titration to be more easily observed.) Dish "B" is used as a control. Dish "A" is placed over the bunsen, on the gauze and, when the contents begin to boil, 0·5 ml. of the phenol phthalein indicator is added. The gas pressure is reduced until the contents of the dish are just simmering. If the medium is acid in reaction there will be no colour, if alkaline deep red to faint pink according to the degree of alkalinity.

Eyre's Scale is the number of millilitres of either N/1 acid or N/1 alkali required to render one litre of medium neutral The scale is graded on a "Plus and Minus" system according to the number of millilitres of free N/1 acid or alkali required to be present in the finished medium.

"0" indicates NEUTRAL.
"+" „ ACID.
"−" „ ALKALINE.

e.g. "−8 on Eyre's Scale" would indicate a medium containing 8 ml. of free N/1 alkali per litre.
"+10 on Eyre's Scale" would indicate a medium containing 10 ml. of free N/1 acid per litre of medium.

Example

A medium is to be adjusted to "+ 10 on Eyre's scale and upon adding the phenol phthalein is found to be acid. N/10 sodium hydroxide solution is run into 10 ml of boiling medium until the "end-point" (very faint persistent pink) is reached.

Burette reading before titration commenced: 1 2 ml.
Burette reading at the "end-point" 6·4 ml
Then 10 ml. of medium required 5 2 ml. of N/10 alkali to neutralize it
and 100 ml. of medium required 52 ml. of N/10 alkali
1,000 ml. of medium requires 52 ml. of N/1 alkali.

But the medium is to be "+10" on Eyre's scale, therefore 42 ml. of N/1 sodium hydroxide must be added, leaving the equivalent of 10 ml. of N/1 hydrochloric acid free in each litre.

Preparing Standard Solutions for Adjusting Media

Normal sodium carbonate (a standard for adjusting other solutions actually used)

Heat approximately 85 grams of A.R. sodium bicarbonate to dull red heat in a platinum crucible for at least ten minutes, then place in a desiccator over fused calcium chloride until

cold. Weigh rapidly and wash the contents of the dish into a clean beaker. As soon as the salt has dissolved make up to:

$$\frac{\text{weight of salt}}{53} \times 1{,}000 \text{ ml. of distilled water}$$

Note. If a platinum dish is not available, a porcelain evaporating basin will serve, but this must be heated for a longer period of time.

This solution must be very carefully prepared as it forms the standard by which subsequent solutions will be adjusted.

Normal hydrochloric acid solution

Make up 100 ml. of pure hydrochloric acid (S.G. 1·16) to 1 litre with distilled water. Titrate against the standard sodium carbonate solution:

Measure exactly 25 ml. of standard sodium carbonate solution into a 100-ml. conical flask and add a few drops of methyl orange indicator (0·1 per cent. solution). Fill a 50-ml. burette with the hydrochloric acid solution. Run the HCl into the flask, a little at a time, until the indicator just changes colour. Note the burette reading. Refill the burette, empty and wash the flask out with distilled water and repeat the titration taking great care with adding the acid as the previous "end-point" is reached. Again note the reading.

E.g.,

1st burette reading	Zero
2nd " "	25·4
3rd " "	25 7

Take the average of the two readings—which would be 25·55—this would indicate that the acid is slightly *weaker* than the soda solution.

Use of "Factors". Instead of attempting to adjust the acid solution to exact normality a "factor" may be used to indicate its exact strength as compared to actual normality.

E.g.:

The acid is slightly weaker than the N/1 caustic soda solution so that 25 ml. of the acid will equal something less than 25 ml. of the soda

To obtain the "factor" then we divide the amount of soda taken by the amount of acid used to neutralize it.

$$\frac{25 \text{ ml (amount of soda)}}{25{\cdot}55 \text{ (amount of acid)}} = 0{\cdot}978 — \text{the factor for the N/1 acid.}$$

and 10 ml. of the acid would be equivalent to 9·78 ml. of N 1 soda.

N/1 sodium hydroxide solution

Weigh out approximately 40 grams of caustic soda (pure) in a porcelain dish and make it up to 1,000 litres with distilled water. Sodium hydroxide (caustic soda) is deliquescent and accurate weighing is difficult so that there is no point in weighing exactly 40 grams.

Place 25 ml. of the soda solution in a 100-ml. conical flask, add a few drops of the methyl orange indicator. Fill the burette with N/1 HCl solution and run the acid into the flask until the indicator just changes colour. Repeat this titration as for the previous one and take the average of your readings.

Eg :

1st burette reading	·	Zero
2nd „ „		25 2 ml N/1 acid used.
3rd „ „	·	25·6 ml. „ „
Average „	· ·	25 4 ml. „ „

On this occasion the soda solution is slightly stronger than the acid and 25 ml. of the soda equals something more than 25 ml. of the acid.

In this instance, too, the " factor " of the acid must be taken into account.

The factor of the N/1 sodium hydroxide solution will be:

$$\frac{25 \text{ (amount of soda used)}}{25\ 4 \times 0\ 978 \text{ (amount of acid used)}} = 0\ 993 — \text{(the factor for the N/1 caustic soda solution)}$$

and 10 ml. of the soda would be equivalent to 10·153 ml. of acid.

Use of Screw-cap Bottles

During recent years the screw-cap bottle has largely superceded the test-tube in the Bacteriological laboratory. It has much to commend it, particularly where media are made in bulk and stored or distributed to other laboratories. The bottles are made of clear white flint glass and, with the exception of the " medical flat " variety, withstand all the treatments meted out to glassware in course of the various sterilization methods. The types of bottles available, together with their

capacities, types of screw-caps and washers are given in the following table:

Bottle	*Capacity*	*Washer*	*Screw-cap*
1 gal.	4,600 ml.	Compo-cork	Special to fit
80 oz.	2,400 ml.	3 mm. rubber	" Charlton " senior
40 oz.	1,190 ml.	3 mm. "	" "
20 oz.	600 ml.	3 mm. "	" "
10 oz.	290 ml.	2 mm. "	" junior
6 oz.	170 ml.	2 mm. "	M3
4 oz.	115 ml.	2 mm. "	M3
2 oz.	60 ml.	2 mm. "	M2
1 oz.	28 ml.	2 mm. "	M3
½ oz.	15 ml.	2 mm. "	M2
1 oz. (universal container)	28 ml.	2 mm. "	Special size
¼ oz. (Bijou)	6 ml.	2 mm. "	M2

The main disadvantage is that they restrict the air supply during incubation of cultures which may result in poor growths of organisms which ordinarily require abundant free oxygen and also, the partial bleaching, for the same reason, of indicators in certain media. This can be obviated by loosening the screw-caps during incubation, but risks of contamination or of spilt cultures are then introduced.

CHAPTER XVIII

FILTRATION OF MEDIA

APART from any consideration of the various filters used for the sterilization of media, filtration plays an important role in its preparation. Various substances may be used, from specially prepared papers (Chardin papers) to cellulose and cotton-wools, and from sand to sintered glass. According to the consistency and amount of deposited matter in media so one or more of the filtration methods should be chosen.

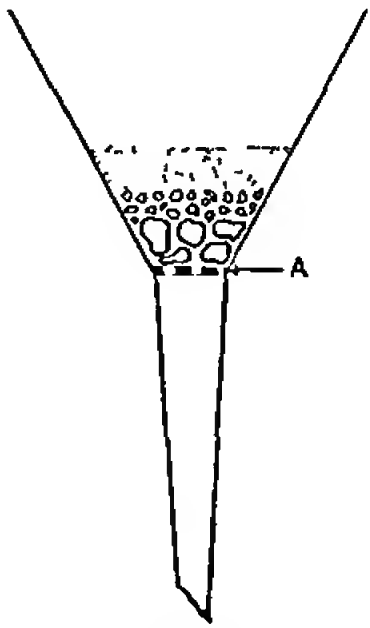


Fig 33
A = Perforated porcelain disc.

For very coarse particles a filter consisting of a large funnel containing a layer of small pebbles followed by layers of sand of varying coarseness may be employed (Fig. 33).

Paper filters

Chardin papers are obtainable commercially and may be used for most of the straightforward filtrations. The papers are folded so that the maximum filtering surface of the paper is held clear of the sides of the funnel

Cellulose wool and "tissue"

For most routine filtrations cellulose tissue is to be recommended. *It is obtainable in rolls and can be readily cut into* convenient sizes as required. The great advantage of the tissue over the ordinary wool (or wadding) is that it is covered back and front with layers of cotton gauze which hold the cellulose wool firm, preventing it from disintegrating in the filter apparatus. This material is particularly suited for use with Buchner funnels for vacuum filtration work.

Cotton-wool

The most important use for cotton-wool as a filtering-agent is plugging tubes and other vessels to prevent the ingress of air-borne bacteria. For this purpose it is essential that non-absorbent wool be used. As cotton-wool consists of many minute fibres intermingled, the minute air spaces between the fibres will hold water if the absorbent variety is used and this water will provide ready access to the interior of the vessel for any organisms which alight on the outer surface of a plug made from it. Cotton-wool is not to be recommended for wet filtrations as it clogs readily and considerable wastage of media results.

High-quality surgeons' wool should be used. Cheaper grades of wool are made up from short fibres which tend to fall into the medium: cotton-wool may be tested for this by taking a small piece and waving it to and fro against the fingers of the other hand over a piece of dark-coloured paper. Short lengths of fibre will fall and the amount can be noted.

Surgeons' lint

Ordinary white surgical lint is useful when filtering the residues from meat-infusion media. A fairly large square of lint is cut and laid in a filter-funnel, allowing a good overlap. The bulk of the media is poured through the filter and then the entire residue may be poured into it. After allowing the moisture to drain through, the lint is folded over the residue and the piece of lint lifted and wrung out into the funnel (care being taken to see that no fragments of residue are squeezed through between the folds).

Asbestos fibre

Fine asbestos fibre is washed in dilute hydrochloric acid and then in distilled water. A thick emulsion (it should just pour from a wide-necked bottle) is made in distilled water and kept for use as required. For filtration a good-quality filter-paper (Whatman or similar) is placed over the perforations of a Buchner funnel and a small quantity of the emulsion poured

over it. The Buchner funnel is fitted into a filter-flask, connected to the vacuum pump and air drawn through the system until the fibre settles down into a firm even disc of approximately ¼ in. thickness. Another filter-paper is placed over this and air drawn through again until no more water is extracted. The filter is then ready for use.

Paper-pulp

An emulsion of filter-paper clippings is prepared as for asbestos fibre and used in a similar manner. (*Filter-paper clippings are obtainable commercially*)

Sintered glass

Such a wide range of porosities can be obtained in these filters that many of them may be classified amongst the bacterial filters. The coarser filters may be employed for filtration of fluid media which require a high standard of clarity.

Sand and paper

A useful filter for removing coarser particles from media, prior to final filtration, can be made in the laboratory and installed as a more or less permanent piece of apparatus It consists of a length of wide-bore glass tubing (approximately

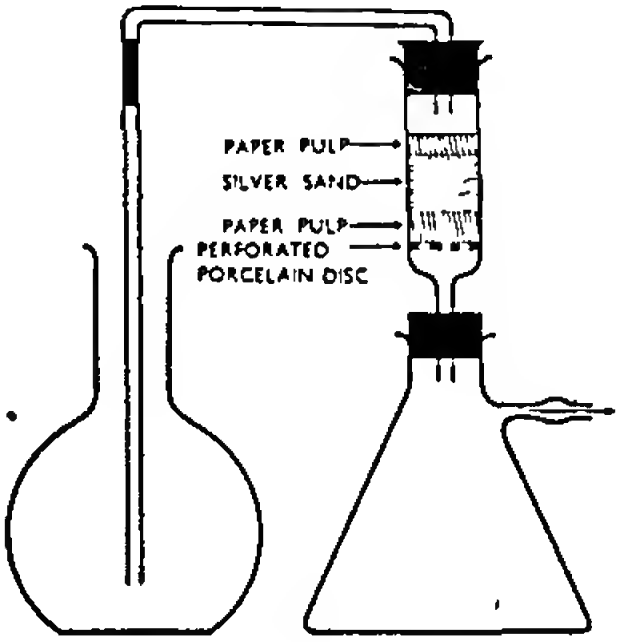


Fig 34

40 mm.) drawn out at one end to a tube of approximately 10 mm. bore. A perforated porcelain disc is inserted and pushed to the drawn out end. Against this disc paper or asbestos emulsion is packed to a depth of ½ in. Next a layer of silver sand is poured in to a depth of 3 in. followed by another layer of paper or asbestos emulsion. Into the wide end of the tube a rubber bung through which passes a short length of 5 to 6 mm. bore tubing is fitted. The method of use is as shown in Fig. 34. At the completion of each filtration the tube is disconnected, emptied, cleaned and refilled as before in readiness for re-use. Large volumes of media can be dealt with in this manner and final filtrations will be speeded up by the removal of much of the coarser deposits which would otherwise clog the more exacting filters.

CHAPTER XIX

CULTURE MEDIA

Normal saline solution (physiological salt solution)

Sodium chloride	8·5 grams
Distilled water	1,000 ml.

Some workers prefer to use 9·0 grams of sodium chloride, but there is little point in this variation. Test-tubes containing approximately 10 ml. quantities, plugged and sterilized are useful at the work-bench.

Peptone water

Peptone	10 grams
Sodium chloride	5 „
Tap water	1,000 ml.

The peptone and salt are mixed into a thin paste with a little of the water and the remainder of the water added. The mixture is placed in the steamer to dissolve (this usually takes twenty to thirty minutes).

For general use the reaction is adjusted to pH 7·6 as subsequent heating will reduce the reaction to pH 7·2–7·4. The solution is returned to the steamer for thirty minutes, filtered, and tubed off in 5 ml. quantities and either autoclaved or fractionally sterilized at 100° C. for twenty minutes.

For use in the CHOLERA RED REACTION Fairchild's peptone is used and the solution adjusted to pH 8 (becoming approximately pH 7·8 after sterilization).

Peptone water for sugars

As a base for sugar media the reaction of ordinary peptone water is adjusted to pH 8 after which it is steamed for twenty minutes and filtered. 1 per cent. Andrade's Fuchsin Indicator is added and the peptone water flasked (or bottled) off in 100 ml quantities (or larger if necessary). To each flask or bottle 1 per cent. of the sugar required is added, with the exception of the more rare sugars such as Dulcitol or Rhamnose when ½ per cent. is used. The sugar solutions are again filtered

and tubed off into small test-tubes or screw-cap bottles containing inverted Durham tubes. Coloured wools are usually used to distinguish between the various sugars and the tubes are plugged with wools of the appropriate colours or screw-caps painted with "dabs" of colours (see page 178). They are then fractionally sterilized at 100° C. for fifteen minutes.

The greatest possible care must be taken to ensure that no error is made with labelling the various sugars and it is essential to remove all traces of each sugar from all pieces of apparatus used before proceeding with the next. The medium should be quite clear and, in bulk, of an amber colour when cool but, whilst in the steamer and until cooled to below 70° C. it should be bright pink.

Some workers prefer to sterilize all sugar solutions by filtration, bottling or tubing off into sterile vessels, observing sterile precautions throughout the operation. Special hooded fillers for this purpose can be purchased.

Neutral red (as in L.B.A.) or Phenol red may be used in place of Andrade's indicator.

Meat-extract broth (Liebig's or Lemco)

Liebig's meat extract (Lemco)	5 grams.
Peptone	20 grams.
Sodium chloride	5 grams.
Tap water	1,000 ml.

The meat-extract is weighed out on a clean piece of paper, counterbalancing this paper with a similar piece on the opposite scale-pan. The piece of paper with its meat-extract is placed with the other ingredients in a flask and heated in the steamer for twenty minutes. The reaction is adjusted to pH 7·6 and the flask returned to the steamer for a further twenty minutes. The broth is then filtered through ordinary filter-paper. For keeping as stock, the broth is flasked or bottled off in 100 ml. or 250 ml. quantities and fractionally sterilized at 100° C. for twenty minutes.

Trypsinized ox-heart broth

All fat and connective tissue is removed from fresh ox-hearts and the lean meat finely minced (if cut into long narrow strips

the meat can be " fed " into the mincer with minimum trouble). To each 500 grams of the minced heart 1,000 ml. of tap water is added and the mixture allowed to macerate overnight (i.e. allow the water to dissolve out the natural juices from the muscle fibres). Most of these juices would be sealed in the meat if the mixture was subjected to heating straight away because the outer surface of each fragment of meat would be coagulated Next morning the mixture is slowly heated to 75° C., then allowed to cool to 37° C. and just sufficient 20 per cent. caustic soda solution added to render it slightly alkaline to litmus. Next Liquor Trypsini Co (Allen and Hanbury) is added in the proportion of 1 per cent. and the mixture is placed in the 37° C. incubator for from three to four hours. Trypsin has the power to digest, or break down proteins into simple organic substances which can be readily utilized as foods by bacteria. It can only act in an alkaline medium, hence the addition of the caustic soda. At the end of three hours the following test is made for the presence of peptone in the broth:

5 ml. of the broth, 5 ml. of N/1 NaOH and one drop of 5 per cent. copper sulphate solution are mixed in a test tube. A true pink colour denotes that there is peptone present, whilst a purple-blue colour denotes its absence and the incubation must be continued until a further test gives the pink tint required.

Opinions appear to differ regarding the next stage, some workers render the broth faintly acid by adding a small quantity of acetic acid to stop the action of the trypsin Others leave the broth as it is; believing that subsequent heating whilst the mixture is still alkaline assists in precipitating the " phosphates" that, in the first method, come down at a later stage and prolong the process of filtration. Whichever of the two methods is adopted the mixture is next brought slowly to the boil and allowed to continue boiling for fifteen minutes. It is then left to cool and to stand overnight. Next morning the clear broth is syphoned off and the sediment poured into a piece of lint stretched loosely over a funnel. The lint is folded over the sediment and wrung out into the remainder of the broth. The broth is then made up to its original bulk with water and 0·25 per cent. sodium chloride and 0·125 per cent. calcium chloride added. The reaction is adjusted to pH 7·6, the broth

Lactose bile-salt agar (MacConkey)

Agar-agar fibre (or powder)	25 grams.
Peptone	10 „
Sodium taurocholate	5 „
Tap water	1,000 ml.

The ingredients are dissolved in the water in the steamer (this should take about thirty minutes from the time 100° C. is reached). The reaction is adjusted to pH 7·6 and the medium returned to the steamer for a further twenty minutes. It is then filtered through cellulose tissue and lint.

The filtrate is measured and whilst still hot, 1 per cent. lactose and just sufficient freshly prepared 0·5 per cent. solution of neutral red to give a " claret " tint are added. The medium is tubed off in small quantities (screw-cap bottles holding 100 ml. are convenient) and fractionally sterilized at 100° C. for twenty minutes.

Note.—A slight turbidity in this medium is no great disadvantage, in fact many acid-producing organisms create a distinctly turbid halo round the colonies on plates poured from it.

The greatest care must be taken when sterilizing all media containing lactose as excess heat destroys the lactose and false reactions will result.

Lactose bile-salt fluid medium (MacConkey)

As above, omitting the agar, for SINGLE STRENGTH.

The quantities of ingredients are proportionately increased for DOUBLE STRENGTH. The medium is tubed or flasked in 10 ml. and 50 ml. quantities and inverted Durham's tubes inserted prior to fractional sterilization.

Alizarin-iron-thiosulphate medium (McEwen)

Base Medium

Peptone ("Bacto" or "Oxoid")	20 grams.
Sodium chloride	5 „
Agar	25 „
Water	900 ml.

The ingredients are dissolved in the water at 100° C. in the steamer, the reaction adjusted to pH 8·0 and the medium

returned to the steamer for twenty minutes. It is then filtered through cellulose tissue.

10 grams of lactose and 5 grams of soluble starch (potato) are mixed to a smooth paste with 20 ml. of water and 80 ml. of boiling water added, stirring continuously. This is added to the bulk of the medium which is then bottled off in 50 ml. quantities and fractionally sterilized at 100° C. for twenty minutes. *Note.* The base medium must not be filtered after the addition of the starch.

Solution A

Ferric citrate (scales)	2 grams.
Alizarin red "S"	0·2 „
Water	100 ml.

The ingredients are dissolved in the water in the steamer and then stored in a screw-cap bottle. It keeps well.

Solution B

Sodium thiosulphate	10 grams.
Water	100 ml.

The solution is steamed for twenty minutes and stored in a screw-cap bottle.

Solution C

Sodium deoxycholate	2·0 grams.
Water (warm)	100 ml.

The solution is stored in a screw-cap bottle. It may be sterilized for one hour in the 60° C. water-bath.

When required for use the base-medium is melted and the following added to each 50 ml.

Solution A	2·5 ml.
Solution B	2·5 „
Solution C	0·2 „

The mixture is then cooled to 45–50° C. and plates poured

Using "National Collection" Type Cultures the following results have been constant over a series of batches of the medium:

E. coli	Feebly-growing bright red-violet colonies approximately 1 mm in diameter at 24 hours at 37° C., or no growth.
E. fruendii	Similar to above but slightly more vigorous in growth.
Alkaligenes faecalis	Large lilac-coloured colonies with pale irregular margins.

Salmonella typhi	Pale pink effuse colonies, developing a small black central spot after 24 hours at 37° C.
Salmonella schottmülleri (and allied organisms such as S. paratyphimurium, etc.)	Opaque red colonies with a large black central zone. This black zone appears as a concavity with the red outer edge of the colony forming a distinct "bevel".
Shigella paradys. (Flexner)	Small opaque, pink colonies with entire edges at 24 hours, but tending to irregularity and brighter pink colour on standing at room temperature.
Shigella paradys. (Sonnei)	Semi-translucent, pale pink effuse colonies *with definitely nippled centres.*
Shigella alcalescens	Bright pink umbonate colonies with edges which appear to diffuse into the medium.

In the writer's opinion the use of sodium deoxycholate in quantities which completely inhibit the growth of the "normal" intestinal flora introduces the risk of inhibition of the abnormal organisms too, should a slight excess of the salt be inadvertently added (there is little or no opportunity for ready appreciation of such a contingency). The formula given merely aims at slowing down the development of coliform colonies and was decided upon following a series of experiments using a wide range of dilutions of sodium deoxycholate. Dilutions lower than that employed above appear to show a tendency to retard the development of colonies of some of the pathogenic organisms.

Note.—Further work upon the above is being carried out; the formula is introduced here in the hope that readers may be interested to the point of trying it in parallel with methods at present in use and communicating their observations to the author.

Litmus lactose bile-salt agar

Agar	25 grams.
Peptone	20 „
Lemco (Liebig's extract)	5 „
Sodium taurocholate	5 „
Sodium chloride	5 „
Tap water	1,000 *ml.*

The ingredients are dissolved in the water in the steamer and then cleared with white of egg (see "Nutrient Agar", page 152). The reaction is adjusted to pH 7·6 and the medium

returned to the steamer for thirty minutes, after which it is filtered through cellulose tissue and lint. To the hot filtrate 2 per cent. lactose and sufficient 5 per cent. litmus solution to give a deep blue colour are added. The medium is tubed off in 40 ml. quantities and fractionally sterilized at 100° C. for fifteen minutes. Overheating bleaches the litmus.

Lactose fermenting organisms give red colonies.
Non-lactose fermenters give blue or colourless colonies.

Lactose-tellurite agar

Agar	20	grams.
Peptone	10	„
Lactose	5	„
Di-potassium hyd. phosphate	2	„
Sodium chloride	5	„
Water	1,000	ml

The ingredients, with exception of the lactose, are dissolved in the water in the steamer and then filtered Whilst the solution is still hot the lactose is added and the medium tubed off in 15 ml. quantities and fractionally sterilized at 100° C. for twenty minutes. When required for use the medium is melted and 1 ml. of a 1:1,000 solution of potassium tellurite added to each 15 ml. tube, mixed thoroughly and plates poured.

Endo's medium (modified)

A quantity of nutrient agar is melted and 0·6 per cent. of a hot 10 per cent. solution of sodium carbonate and 1 per cent. lactose (previously dissolved in a few ml. of hot water) are added and thoroughly mixed. 0·5 per cent. of a saturated alcoholic solution of basic fuchsin is added and again the medium is well mixed. Next 2·5 per cent. of a hot 10 per cent. solution of sodium sulphite (cryst) is added and the medium vigorously shaken to ensure that all ingredients are evenly distributed. It is then tubed off in 40 ml. quantities and fractionally sterilized at 100° C. for twenty minutes.

Lactose fermenting organisms—red colonies on a straw-coloured background.
Non-lactose fermenters give no change in colour.

Conradi-Drigalski medium (modified)

A quantity of nutrient agar is melted and 1 per cent. nutrose (dissolved in a few ml. of slightly alkaline hot water) is added. Sufficient litmus solution to make it 13 per cent. of the bulk when added to the agar is placed in a separate flask and heated in the steamer for five minutes. To the litmus solution sufficient lactose to make it 1·5 per cent. of the bulk is added. Whilst both the nutrient agar and the litmus-lactose solution are still hot they are mixed and 0·2 per cent. of hot 10 per cent. sodium carbonate solution and 1 per cent. of 0·1 per cent. crystal violet (freshly prepared) are added and the medium mixed thoroughly. It is then tubed off in 40 ml. quantities and fractionally sterilized at 100° C. for twenty minutes.

Lactose fermenting organisms—red on violet background.
Non-lactose fermenters—blue on violet background.

Eosin-methylene blue agar

Base :

Peptone	10 grams.
Di-potassium phosphate	2 „
Agar	25 „
Distilled water	1,000 ml.

The ingredients are dissolved in the water in the steamer and the reaction adjusted to pH 7·6. The medium is then returned to the steamer for thirty minutes, after which it is filtered through cellulose tissue and lint. It is bottled off in 100 ml. quantities and autoclaved.

When required for use, 100 ml. is melted and the following ingredients added :

Lactose	1 gram.
Eosin (watery) 2 per cent. solution	2 ml.
Methylene blue (0·5 per cent. solution)	1·25 „

The mixture is returned to the steamer for twenty minutes, cooled to 50° C. and plates poured.

Urea agar

Urea	20	grams.
Glucose (anhyd.)	1	gram.
Peptone ("Bacto" or "Oxoid")	1	„
Potassium hyd. phosphate	2	grams.
Sodium chloride	5	„
Agar	20	„
0·02% Phenol red solution	15-20	ml.
Distilled water	900	„

All ingredients, except the urea, are dissolved in the water at 100° C. in the steamer and the reaction adjusted to pH 7·4. The medium is returned to the steamer for thirty minutes, filtered through cellulose tissue and lint and distributed into test-tubes to provide slopes with deep " stubs " when eventually sloped for use. The urea is dissolved in 100 ml. of distilled water and sterilized by filtration (Seitz). When required for use, tubes of the medium are melted, 0·5 ml. of the urea solution added to each and the tubes sloped. Urea-splitting organisms (i.e. proteus) produce a pink coloration of the medium within two hours when incubated at 37° C. in a water-bath. If inoculated with mixed cultures some coloration of the surface of the medium may occur ; but when proteus organisms are present the entire medium rapidly becomes red.

Koser's citrate medium

Sodium ammonium phosphate	1·5	grams.
Potassium acid phosphate	1·0	„
Magnesium sulphate	0·2	„
Sodium citrate	3·0	„
Distilled water	1,000	ml.

All ingredients are dissolved in the water, tubed off in 10 ml. quantities and autoclaved.

Dieudonne's medium (for V. cholerae)

Fresh ox or sheep's blood is defibrinated (this can be done either with glass beads in the bottle and shaking the blood

vigorously immediately upon collection or with a sterile egg-whisk in an open-mouthed jar). An equal volume of N/1 NaOH is added, well mixed with the blood and the mixture steamed for thirty minutes. To every seven volumes of melted nutrient agar three volumes of this alkaline blood mixture is added. The medium is tubed off in 40 ml. quantities and fractionally sterilized at 100° C. for twenty minutes.

Note.—Poured plates should be thoroughly dried for twenty-four hours in the 37° C. incubator before use.

Goldberg's alkaline egg medium (for V. cholerae)

To one egg, broken into a beaker, an equal volume of distilled water is added (to find the volume of the egg place it into a measuring cylinder which has been partially filled with water and the volume of the water read before and after adding the egg). A volume of 6·5 per cent. solution of sodium carbonate equal to the combined volume of the egg and water is next added and the mixture steamed for thirty minutes. A quantity of nutrient agar is melted and 1 per cent. sucrose added. To each five volumes of the agar, one volume of the alkaline egg mixture is added and flasked or bottled off and fractionally sterilized at 100° C. for twenty minutes.

Glucose broth

1 per cent. glucose is added to ordinary nutrient broth and the reaction adjusted to pH 7·6. The medium is steamed and re-filtered and then tubed off in 10 ml. quantities and fractionally sterilized at 100° C. for twenty minutes.

Special glucose broth (for methyl red reaction)

Peptone	0·5 grams.
Glucose (A.R.)	0·5 „
Potassium hyd. phosphate	0·5 „
Distilled water	100 ml.

Tubed off in 10 ml. quantities and fractionally sterilized at 100° C. for twenty minutes.

Pea-flour agar

Pea-flour extract

Pea-flour (Pearce Duff)	100	grams
Sodium chloride	100	,,
Water	1,000	ml.

The pea-flour is infused in the water and sodium chloride in the steamer for twenty minutes and then filtered through cellulose tissue and lint The filtrate is the extract.

To salt-free nutrient agar 5 per cent. of the pea-flour extract is added, the reaction adjusted to pH 7·6 and the medium re-filtered. For stock the medium is flasked or bottled in 250 ml quantities and for use, in 40 ml. quantities and fractionally sterilized at 100° C. for twenty minutes. When required the 40 ml. tubes are melted and plates poured

Note.—The writer has obtained very good results from media prepared from soya flour in place of the pea-flour.

Hartley's Broth

Ox-heart or beef (free from fat and minced)	1,500	grams.
Tap water	2,500	,,

The minced heart is mixed with the water and steamed slowly to 80° C. and 2,500 ml. of 0 8 per cent sodium carbonate (anhyd) solution added. It is cooled to 40° C. and 50 ml. of pancreatic extract and 50 ml. chloroform added. The mixture is incubated at 37° C for six hours, stirring frequently. 40 ml. of pure hydrochloric acid is added and the mixture steamed for thirty minutes, then filtered through cellulose tissue and lint. The reaction is made neutral to phenolphthalein with N/1 NaOH. The medium is then bottled off in 250 ml. quantities and steamed for twenty minutes. When cool 0·25 per cent. chloroform is added and the medium stored in the cold. When required for use it is steamed for twenty minutes to remove the chloroform, the reaction adjusted to requirements and filtered.

Pancreatic extract (Cole and Onslow)

Fresh pigs' pancreas (fat-free and minced)	500 grams.
Distilled water	1,500 ml.
Absolute alcohol	500 „

The ingredients are shaken together in a large well-stoppered bottle and allowed to stand for three days at room-temperature, shaking occasionally. It is then strained through muslin and filtered through Chardin paper (or cellulose tissue and lint). The filtrate is measured and pure hydrochloric acid added in the proportion of 0·1 per cent. of the bulk. It is then filtered to remove the cloudy precipitate which forms and stored in stoppered bottles. If required for immediate use there is no need to add the hydrochloric acid as this is only added to retard the tryptic action of the mixture.

Trypsinized serum-tellurite-copper sulphate agar (Allison and Ayling)

(A) 2 per cent. nutrient agar-adjusted to pH 8 (using Hartley's broth, Lemco or nutrient meat extract broth plus 1 per cent. peptone).

(B) 10 per cent. copper sulphate (A.R.) solution in distilled water.

(C)

Sterile horse-serum	100 ml.	Filtered through a Seitz filter.
Liq. trypsin co.	8 „	
2 per cent. pot. tellurite solution	10 „	

(*Note.*—If using "Injectio Trypsini" and steam-sterilizing the pot. tellurite solution prior to adding, the filtration through the Seitz filter may be dispensed with.)

To each 100 ml. of the nutrient agar previously melted and cooled to 50° C., 10 ml. of "C" and 0·5 ml. of "B" are added. Plates are poured and dried off in the 37° C. incubator.

Sugar-free broth (Cole and Onslow)

"Laitproto"	200 grams.
Sodium carbonate	20 „
Distilled water	2,000 ml.
Pancreatic extract (see above)	100 „

The "*Laitproto*" powder is sprinkled over the surface of a litre of water in a large dish, constantly stirring and applying gentle heat. A boiling solution of the 20 grams of sodium carbonate in the remaining litre of water is added slowly. When thoroughly mixed it is poured into a Winchester quart bottle, cooled to 40° C. and 50 ml. of the pancreatic extract and 15 ml. of chloroform added. After shaking vigorously it is incubated for five days, repeating the shaking frequently. A further 50 ml. of the pancreatic extract is then added to the mixture which is again shaken vigorously and the bottle returned to the incubator for a further ten days. It is next transferred to a 4-litre flask, 400 ml. of N/1 hydrochloric acid added and steamed for one hour, followed by filtration through Chardin paper (or cellulose tissue and lint).

For use, two volumes of 0·5 per cent. sodium chloride is added to one volume of this digest, adjusted to pH 7·6 and sterilized in the steamer. If storing for future use, the stock digest may be kept in bulk by adding 0·25 per cent. chloroform When required for use it is steamed for twenty minutes to remove the chloroform.

Note.—Recent war-time experience has proved that the following substitute gives reasonably good results.

"National dried milk powder"	200	grams.
Sodium chloride · · ·	20	„
Distilled water · · ·	2,000	ml.
Liquor trypsini co. · ·	100	„

Proceed as before, incubating for the full fifteen days.

Löffler's serum slopes (modified). (See "Collection of Serum", page 179.)

(A) Horse serum · ·	1,000	ml.
(B) 10 per cent. Lemco 2 per cent. peptone 0·5 per cent. NaCl	30	„
(C) N/1 HCl · · ·	15	„
(D) 10 per cent. glucose solution	30	„

The ingredients are mixed and tubed off in quantities to make slopes, the bases (or thick end) of which should well cover the bottoms of the tubes. The tubes are sloped in the inspissator and heated to 75°–80° C. until coagulated and firm. The slopes are then packed loosely in wire baskets and steamed at just below boiling point on the remaining two successive days of fractional sterilization. Immediately after the final sterilization the slopes are removed from the steamer and allowed to cool rapidly to ensure plenty of "water of condensation".

Note.—To obtain the horse serum, to each litre of fresh horse-blood 10 ml. of 10 per cent. potassium oxalate is added, mixed and the red-blood corpuscles allowed to settle. The plasma is syphoned off and to each litre of plasma 22·5 ml. of 4 per cent. calcium chloride solution is added, and the plasma shaken to separate the fibrin.

Löffler's serum slopes (modified)

Horse, sheep or ox-serum	300	ml.
Nutrient glucose broth	100	„
Glycerine	40	„

The ingredients are mixed and tubed off as above and the tubes sloped in the inspissator and heated to 75°–80° C. until coagulated. The slopes are then fractionally sterilized and cooled rapidly as above.

Note.—Some workers prefer to omit the glycerine, whilst others use plain nutrient broth in place of the glucose broth.

Lowenstein's egg medium

(A)	Magnesium sulphate	0·5	grams.
	Potassium acid phosphate	0·5	„
	Sodium dihydrogen citrate	0·5	„
	Asparagine	1·5	„
	Water	500	ml.
	Glycerine	50	„
	Heat to dissolve.		
(B)	Of mixture (A)	320	ml.
	Pure potato flour	12	grams.

(Groulty : Cross & Blackwell.)

The flour is mixed to a smooth paste with the solution and the mixture autoclaved at 15 lb. for twenty minutes.

A number of fresh eggs are scrubbed and soaked in methylated spirit for one hour. Into mixture (B) eight whole eggs and the yolks of two are added, whilst the mixture is maintained at a temperature of 50°–55° C. After mixing thoroughly, avoiding bubbles, the mixture is tubed off (U.G.B. screw-cap bottles, 3½ × 1 in.), sloped in the inspissator and heated at 85° C. for thirty minutes. The inspissation (at 75° C.) is repeated for thirty minutes on the following day.

Dorsett's egg medium

The shells of four new-laid eggs are sterilized by pouring methylated spirit over them and allowing them to dry With a sterile scalpel the shell is broken at one end of each egg without damaging the inner membrane and a small hole through both shell and membrane pierced in the other end. The contents of the eggs are then blown into a sterile flask containing 25 ml. of water in which a few glass beads have been placed. The plug of the flask is replaced and the contents shaken vigorously. The mixture is then poured through sterile surgical gauze laid in a funnel and tubed off into sterile tubes or screw-cap bottles (see above) and fractionally inspissated at 75° C. for three days.

Petroff's medium

500 grams of finely minced beef or veal is allowed to macerate in 500 ml. of 15 per cent. glycerin solution for twenty-four hours after which the mixture is strained through lint. To the filtrate gentian violet is added in the proportion of 1 to 5,000 and the mixture steamed for thirty minutes. Fresh eggs are soaked in methylated spirit and allowed to dry ; then, using similar technique as for Dorsett's egg medium (see above), the contents of the eggs are blown into a large sterile beaker or flask. The volume of egg-contents is measured and added to the glycerine-meat-violet solution in the proportion of two parts of egg to one part of solution and thoroughly mixed. The mixture is then tubed off, sloped in the inspissator and

heated slowly so that it reaches a temperature of 85° C. in approximately one-and-a-half hours. The slopes are sterilized at 75° C. for one hour on the two succeeding days.

Boeck and Drbohlav's medium (for amoebae)

Four fresh eggs are washed and the shells sterilized with methylated spirit. The contents are blown into a sterile flask (as for Dorsett's medium), 50 ml. of Locke's solution added and mixed thoroughly. The mixture is run into sterile test-tubes or screw-cap bottles to form short slopes about 1½ in. long with a good solid base to them. The tubes or bottles are sloped in the inspissator and coagulated at 70° C. after which they are sterilized in the autoclave. A mixture of eight parts of sterile Locke's solution to one part of sterile human serum, inactivated at 55° C. for half an hour, is run into the tubes until each slope is just covered. All tubes are incubated to test for sterility and contaminated tubes discarded.

1 per cent. solution of dried egg albumin in Locke's solution sterilized by passage through a Seitz filter may be used in place of the serum. The reaction of the medium should be between pH 7·2 and pH 7·8 and should not need adjusting.

Dobell and Laidlaw's modification. Sterile horse-serum is sloped as for the egg medium and coagulated in the inspissator at 80° C. The slopes are covered with serum or egg albumin in Locke's solution as before. A small quantity of rice starch (sterilized in thin layers at 180° C. for one hour) is added to each tube.

Locke's modification of Ringer's solution

Sodium chloride	1·8 grams.
Calcium chloride	0·048 ,,
Potassium chloride	0·084 ,,
Sodium carbonate	0·05 ,,
Dextrose	0·2 ,,
Distilled water	200 ml.

(the reaction is adjusted, if necessary, to pH 7·6).

Alkaline egg medium (for anaerobes)

The whites of two eggs and the yolk of one are beaten in a large beaker and 6 ml. of N/1 NaOH slowly added, stirring the whole time. The volume of this mixture is made up to 500 ml with distilled water, after which it is steamed for one hour. It is then filtered through cellulose tissue and lint and tubed off in 10 ml. quantities, a shallow layer of liquid paraffin is run on the surface of each tube and the medium fractionally sterilized at 100° C. for twenty minutes.

Hard-boiled egg medium (for anaerobes)

Hard-boiled eggs are shelled and small cubes cut from the whites only. One cube is added to each of a number of tubes of nutrient broth, a layer of liquid paraffin added and the tubes fractionally sterilized at 100° C. for twenty minutes.

Thioglycollate medium for anaerobes (Brewer)

Pork infusion solids	10	grams.
Peptone (Thio)	10	,,
Sodium chloride	5	,,
Sodium thioglycollate	1	,,
Agar	0·5	,,
Dextrose	0 to 10	,,
Water	1,000	ml.

Methylene blue is added to make it 1.500,000 of the bulk. The medium is tubed off in 10-12 ml. quantities in 6 × ⅝ in. tubes and autoclaved at 15 lb. for twenty minutes.

Cooked meat medium (for anaerobes)

500 grams of finely minced fat-free bullock's heart is boiled in 500 ml of N/20 NaOH solution for twenty minutes. (The pH of this liquor should be approximately 7·5.) The liquor is drained off and the meat spread out to partially dry.

About ½ in. depth of this meat is placed into test-tubes and 10 ml. of peptone water run into each tube The surface of the medium in each tube is covered with a layer of liquid paraffin and the tubes autoclaved at 15 lb for twenty minutes.

Glycerin potato slopes

A number of large well-formed potatoes are scrubbed and, with a cork-borer or fruit-corer, cylindrical pieces cut from them. The cylinders are cut diagonally to form two wedges (or slopes) from each piece and the wedges soaked in 1 per cent sodium carbonate solution for one hour. They are then placed in test-tubes into the bottom of each of which has been pressed a small wad of cotton-wool soaked in 5 per cent. glycerin solution. The tubes are plugged with cotton wool and fractionally sterilized at 100° C. for twenty minutes. At the end of the third sterilization, and when the tubes have cooled, the cotton wool plugs are cut off level with the tops of the tubes and warm petroleum jelly run on to seal the tubes and so prevent undue evaporation of the contents. Screw-cap bottles may be used instead of plugged tubes.

Crystal-violet potato (Corper)

Corper adds 1·75,000 crystal violet to the 1 per cent. sodium carbonate solution and soaks the wedges of potato for two hours, sterilizing the finished medium by autoclaving at 15 lb. pressure for twenty minutes.

Wilson and Blair's medium

(A)	Bismuth citrate	30 grams.
	Distilled water	25 ml.
	Ammonium hydroxide (·880)	10 ,,

The ingredients are mixed and made up to 250 ml. with distilled water.

(B)	Sodium sulphite (A.R.)	120 grams.
	Distilled water	300 ml.

To this solution 150 ml. of (A) is added and the mixture warmed.

(C)	Sodium phosphate	30 grams.
	5 per cent. glucose solution	150 ml.

This is dissolved by warming slightly, added to the "A plus B" solution and then boiled for two minutes.

For stock, solutions (A), (B) and (C) are kept in stoppered bottles.

To prepare solid medium ·

Trypsinized ox-heart broth agar · · · · ·	200	ml.
A-B-C mixture · · · ·	40	„
8 per cent. ferric chloride solution · · · · ·	2	„
1 per cent brilliant green solution · · · · · ·	0·25	„

The ingredients are mixed and boiled for two minutes, cooled to 60° C. and plates poured.

To prepare liquid medium ·

A-B-C mixture · ·	30	ml.
Nutrient broth · · ·	50	„
Peptone water (Difco) · ·	100	„
8 per cent. ferric chloride solution · · · · · ·	1·5	„
1 per cent. brilliant green solution · · · · · ·	0·1	„
Absolute alcohol · · ·	3	„

After mixing, the medium is tubed off in 10 ml. quantities.

For use, inoculated tubes are incubated at 37° C for thirty hours and the cultures plated out on the solid medium.

Desoxycholate-citrate agar (Leifson)

Preparation of the Pork infusion. 333 grams of fresh lean pork, finely minced are allowed to macerate in 1,000 ml. of water for one hour and then 3·3 ml. of N/1 hydrochloric acid added and the mixture boiled for one minute. It is next filtered through lint, 3·3 ml of N/1 NaOH added, the filtrate made up to its original bulk and boiled for another minute. 10 grams of peptone are added, the reaction adjusted to pH 7·6, the mixture boiled for three minutes and again filtered through cellulose tissue.

To prepare the "Base" medium

Pork infusion · · · ·	1,000	ml.
Agar · · · · · ·	20	grams
Lactose · · · · ·	10	„
Neutral red (1:50,000) solution	20	ml.

The agar is dissolved in the pork infusion in the steamer and the reaction adjusted to pH 7·6. The lactose and neutral red solution are then added whilst the medium is still hot and the medium bottled off in 100 ml. quantities and fractionally sterilized at 100° C. for twenty minutes.

Solution "A"

Sodium citrate	25 grams.
Ferric ammonium citrate	2 „
Distilled water	100 ml.

Solution "B"

Sodium desoxycholate	5 grams.
Distilled water	100 ml.

These solutions are kept in stoppered bottles in the refrigerator. When required for use the solid "Base" medium is melted and to each 100 ml., 5 ml. of each of solutions "A" and "B" are added, mixed thoroughly and plates poured.

Coliform organisms grow poorly and such colonies as do develop are pink in colour. Non-lactose fermenters are colourless with, sometimes, a clear area (or halo) round the colonies.

Tetrathionate broth (for isolation of the typhoid group)

Nutrient broth	900 ml.
Calcium carbonate (precip.) sterilized in the hot air oven at 180° C.	250 grams.
60 per cent. sodium thiosulphate (cryst) solution	100 ml.
30 per cent. iodine solution	20 „

The iodine solution is prepared by dissolving 25 grams of potassium iodide in 50 ml. of distilled water, dissolving the iodine in this and adding the remainder of the water. The broth, calcium carbonate and thiosulphate solution are mixed, tubed or bottled (1 oz. screw-caps) off in 10 ml. quantities and sterilized. When required for use 0·2 ml. of the iodine solution is added to each tube or bottle.

Selenite enrichment medium (for isolation of the typhoid group)

Sodium di-selenite	4	grams.
Peptone	5	„
Lactose	4	„
Disodium hyd. phosphate	9·5	„
Sodium dihyd. phosphate	0·5	„
Water	1,000	ml.

The ingredients are dissolved in the water and steam sterilized (do not autoclave). The pH, which should be 7·1, may be adjusted by varying the amounts of the buffer salts.

Litmus milk medium

To each litre of fresh skimmed milk 10 ml. of the following solution is added :

Azolitmin	10	grams.
N/1 NaOH	0·5	ml.
Distilled water	100	„

The medium is tubed off in 5 ml. quantities and fractionally sterilized at 100° C. for twenty minutes.

Hiss' serum-water

Beef or sheep serum	100	ml.
Distilled water	300	„

Heat slowly to boiling in the steamer and steam for fifteen minutes. It may be found that some sera need further dilution to avoid coagulation when heated.

For use as a " Base " for sugar media add 1 per cent. of the various sugars required and a suitable indicator (phenol red or brom-cresol purple, 1 per cent. of a 1 per cent. solution).

Gladstone and Filde's medium (CCY)

(*a*) *Stock hydrolysed casein.* 200 grams of commercial casein is placed in a litre beaker and 170 ml. of pure hydrochloric acid and 110 ml. of distilled water added. The mixture is stirred rapidly with a glass rod before the casein swells and solidifies, and autoclaved at 15 lb. for ¾ hour. It is then cooled and 40 per cent. NaOH added slowly until the mixture is

neutral to litmus paper. A Buchner funnel is fitted with a Whatman No. 1 filter paper and paper pulp emulsion poured into it to a depth of approximately ½ in., and allowed to drain; another Whatman filter paper is placed over the pulp and air sucked through with a vacuum pump until no more water is extracted. The casein mixture is filtered through this, using only moderate negative pressure from the pump. The filtrate is diluted to 1 litre, 1 per cent. chloroform added and shaken vigorously. This solution should be stored in the dark.

(*b*) *Stock tryptic digest of casein.* 200 grams of casein, and 20 grams of sodium carbonate are mixed with 2 litres of warm water in a Winchester bottle and shaken thoroughly. 100 ml. of Cole and Onslow's pancreatic extract (see page 162) and 10 ml. of chloroform are added, the mixture again shaken well and the reaction tested (it should be pH 7·4–7·5). The mixture is incubated for at least sixteen days, shaking at least once each day and testing the reaction frequently during this period. The reaction must be adjusted if necessary. Following the incubation, the mixture is allowed to stand at room-temperature for two days after which it is filtered through cellulose tissue and lint, 15 ml. of hydrochloric acid in 150 ml. of distilled water added and steamed for one hour. It is then cooled, filtered again and the reaction adjusted to pH 7·4. 1 per cent. chloroform is added and the mixture shaken and stored in a Winchester quart bottle in the dark.

(*c*) *Stock yeast extract.* 250 grams of pressed brewer's yeast is broken up into 1 litre of boiling distilled water over a naked flame and stirred continuously until frothing ceases. The yeast extract is filtered through pulp in a Buchner funnel (as for Gladstone and Filde's medium, page 171) and, after adding 1 per cent. chloroform and shaking vigorously, stored in a Winchester bottle in the dark.

(*d*) *Sodium glycerophosphate* kept in the solid state.

(*e*) *Glutamine solution.* A 0·05 per cent. solution of glutamine in distilled water is prepared and kept in the refrigerator. The glutamine crystals should be kept in a desiccator in the refrigerator. As the solution is not stable only small quantities should be prepared.

(*f*) *Sodium lactate solution.* 50 grams of sodium lactate is dissolved in 100 ml of distilled water (or the commercial product may be purchased as a 50 per cent. syrupy solution).

(*g*) *Preparation of the medium* (CCY) 15 grams of powdered agar is mixed into a smooth paste with 850 ml. of distilled water and steamed for five minutes after which the mixture is run up in the autoclave to 15 lb. pressure and the heat turned off. The mixture is removed from the autoclave at the end of half an hour.

Mix together :

Hydrolysed casein solution	35	ml.
Tryptic digest of casein	15	,,
Yeast extract	100	,,
Sodium glycerophosphate	10	grams.
Sodium lactate solution	10	ml.

The mixture is shaken to dissolve the glycerophosphate, then heated to boiling point and mixed with the hot agar solution. 3·6 ml. of 40 per cent NaOH is added and the mixture returned to the steamer which should not be below 95° C (a precipitate forms and should be allowed to settle) It is then filtered rapidly through a previously heated Buchner funnel (fitted as described on page 172) taking care not to disturb the precipitate that has formed and settled to the bottom of the flask. Immediately filtration is completed (it should be done within ten minutes) 2·05 ml. of pure hydrochloric acid per litre is added. If more than 1 litre of medium is prepared it should be divided into 1-litre quantities and each litre dealt with separately ; the bulk of the medium being retained in the steamer to keep hot.

The reaction is checked, adjusted if necessary to pH 7·6, and 5 ml. per litre of the glutamine solution added. The medium is bottled off in small quantities and autoclaved.

To prepare the medium in liquid form (to replace meat-infusion broths) the agar solution is replaced by 850 ml. of distilled water.

It is claimed for this medium that it is a cheap and efficient substitute for all meat-infusion preparations.

Sabouraud's medium

Peptone	1 gram.
Maltose	4 grams.
Agar	2·3 „
Water	100 ml.

The ingredients are dissolved in the water in the steamer, the medium cleared (as for ordinary agar) and the reaction adjusted to pH 5. It is then tubed off to make slopes for immediate use, or bottled in 250 ml. quantities for stock, and fractionally sterilized at 100° C. for twenty minutes.

Pure blood medium

Rabbit or human blood is tubed off in small sterile tubes and heated at 56° C. for thirty minutes. The medium is useful for maintaining the virulence of streptococci and pneumococci.

"Chocolate" blood agar (MacLeod)

1 lb. of minced and fat-free meat is allowed to macerate in 1 litre of a solution containing 1 per cent. peptone and 0·2 per cent. sodium hydrogen phosphate for forty-five minutes at 60° C. after which it is steamed for thirty minutes. The reaction is adjusted to pH 7·4. 1·3 per cent. agar is dissolved in the medium in the steamer. When required for use 10 per cent. heated blood is added to the molten agar and plates poured. The plates are incubated in a jar containing 8 per cent. carbon dioxide for eighteen hours followed by a further twenty-four hours in the air.

Note.—For use in the "Oxidase Reaction" gonococcus colonies, which are convex translucent and of medium size, turn a bright purple in colour upon flooding the plate with 1 per cent. solution of tetra-methyl-p-phenylenediamine hydrochloride and draining off immediately.

(N. meningitidis is said to give this reaction also.) If sub-cultures are required, selected colonies should be transplanted immediately the colour appears, within ten minutes at most, as colonies rapidly die following application of the test-solution.

Transport medium for Neisseria (Stuart's solution)

Stock materials:

(1) 90 per cent. thioglycollic acid.
(2) 20 per cent. sodium glycerophosphate.
(3) 1 per cent. calcium chloride.
(4) 0·1 per cent. methylene blue (aqueous).
(5) 0·3 per cent. agar (previously well washed in distilled water)
(6) Sterilized powdered charcoal.

All solutions should be sterilized in the steamer for one hour. The charcoal may be sterilized in the hot air oven or tightly stoppered and autoclaved.

To 760 ml. of the agar solution (melted and kept hot), 0·8 ml. of No. 1, 40 ml of No. 2 and 8 ml. of No. 3 solutions are added. The mixture, whilst still hot, is adjusted to pH 7·4 (using N/1 NaOH) and 1·6 ml of solution No. 4 added. The mixture is returned to the steamer for a few minutes and then distributed into small screw-cap bottles and steamed for a further hour. The medium should be allowed to stand in a cool place for at least twenty-four hours before being brought into use.

To make up "Charcoal swabs" for transporting specimens suspected of Neisserial infections

Bottles of the medium are melted and 1 per cent. of the sterile charcoal added. Swabs, made with neutral cotton-wool, are dipped into the molten medium (keeping the charcoal evenly distributed by frequently rotating the bottle in use), returned to their respective tubes, tightly corked and autoclaved at 15 lb. for fifteen minutes.

Noguchi's medium (for treponemata)

(*a*) Long narrow tubes (approximately 8 in. × ¾ in.) are three-quarters filled with a 2 per cent. solution of agar adjusted to pH 6·5 and sterilized. The agar is cooled to 50° C and sterile ascitic or hydrocele fluid added in the proportion of one part of fluid to two parts of agar. A small piece of rabbit kidney is added to each tube and allowed to sink to the bottom before the agar solidifies. When the medium is set, sterile liquid paraffin is poured over the surface to a depth of about 3 cm.

When required for use the medium is inoculated with a capillary pipette passed to the bottom of the tube.

(b) M'leod and Soga's modification of Noguchi's medium. Test-tubes are fitted with rubber bungs which can be pushed right into the tubes. The rubber bungs are bored with a small hole through which is passed a short length of glass tubing of approximately 3 mm. bore. This tube is drawn out into a fine capillary and bent over at an acute angle at its upper end. The test-tubes are half filled with nutrient broth (pH 7·4) and sterilized. When cooled a small piece of sterile tissue is added to each tube. To inoculate, a small piece of sterile surgeon's gauze is drawn through a sterile glass bead and soaked in the material to be cultured and the bead and gauze dropped into a tube of medium. Ascitic or hydrocele fluid is then run into the tube and the rubber bung pushed down until the fluid just enters the glass tube. The capillary end of the tube is then sealed off in the pilot flame of the bunsen. To obtain samples of the subsequent culture the "capillary" end of the tube is broken off and a capillary pipette introduced.

Noguchi's medium (for leptospira)

To each one part of fresh sterile rabbit's serum add three parts of Ringer's solution (Locke's modification, page 166) made semi-solid with 0·3 per cent. agar, molten and cooled to 50° C. The medium is tubed off in long narrow tubes (as for above) and 1 ml. of citrated blood added to the bottom of each tube. When quite cool a layer of sterile liquid paraffin is poured over the surface of each tube of medium.

"N.N.N." (Nicolle Novy MacNeal) medium

Agar	14 grams.
Sodium chloride	6 ,,
Distilled water	900 ml.

Dissolve the ingredients in the steamer and adjust the reaction to pH 7·4. Tube off in 5 ml. quantities and sterilize. When required for use melt the medium and cool to 50° C., add one third of the volume in each tube of defibrinated rabbit blood, mix thoroughly and slope until the medium is solidified.

Rubber stoppers, or cotton-wool plugs soaked in melted paraffin wax should be used to seal the tubes to avoid undue evaporation of the " water of condensation ".

Maitland and Maitland's tissue medium (for viruses)

Rabbit or hen kidneys are removed aseptically and finely minced To each part of the minced kidney three parts of Tyrode's solution is added and the mixture distributed into small conical flasks to form a thin layer over the bottom of the flasks. The virus to be cultured may be added to the kidney during the mincing process. As various viruses may require specific tissues, these tissues are selected as indicated and treated as for the kidney.

Chick-embryo medium

In place of the kidney (above) embryos from eggs incubated for from nine to twelve days are removed aseptically from the shells and cut into small pieces with sterile scissors. Into each of a number of small conical flasks approximately 0·2 grams of the embryo is placed and 10 ml of sterile Tyrode's solution added The cotton-wool plugs of the flasks are covered with tin-foil. As there is always grave risk of bacterial contamination, inoculated flasks (which are incubated for five days at 37° C.) are always tested for possible bacterial contamination by making stained films from the contents before preparing subcultures from them.

Tyrode's solution

Sodium chloride	8 grams
Potassium chloride	0·2 ,,
Calcium chloride	0·2 ,,
Magnesium chloride	0·1 ,,
Sodium hyd. phosphate	0·05 ,,
Sodium bicarbonate	1·0 ,,
Glucose	1·0 ,,
Distilled water to	1,000 ml.

The solution should be sterilized by filtration The addition of living tissues corrects the reaction.

The Addition of Blood[1] to Nutrient Agar

It is both economical and, in the writer's view, an advantage to prepare "buttered" plates and slopes when making blood agar medium.

For plates. Just cover the bottoms of the petri-dishes with molten agar and allow to solidify. To each of two 15 ml. tubes of molten nutrient agar (cooled to 55° to 60° C.) add approximately 0·5 ml. of sterile defibrinated blood. Having carefully flamed the necks of both tubes mix their contents thoroughly by pouring the blood-agar mixture rapidly from tube to tube several times. Again flame the necks of the tubes and pour a thin layer of the blood-agar mixture over the surfaces of the plain agar plates already poured in the dishes.

For slopes. A similar technique to the above may be employed, sloping small quantities of plain agar in the tubes and re-sloping after adding a small quantity of the blood-agar mixture.

Labelling Media

Mackie and McCartney have proposed the following scheme of colour-labelling for quick recognition of media, particularly the sugar media which have, of necessity, to be bundled into groups. Standardization of colours used is long overdue, as most technicians who have moved from one laboratory to another will agree.

[2] **Sugar media:**

Adonitol . . Silver	Glycerol . . Brown and White
Aesculin . . Brown	Glycogen Blue and Yellow
Arabinose Black and Yellow	Inositol . . Gold
Dextrin . . Red and Mauve	Inulin . . . . Yellow and White
Dulcitol . . Pink	Lactose . . Red
Erythritol Black and Red	Laevulose Yellow
Galactose Mauve and White	Maltose . . Blue and white

[1] *Messrs. Burroughs Wellcome offer a regular sterile blood service which obviates the bleeding of laboratory animals for culture-media preparation.*

[2] *Taken from* An Introduction to Practical Bacteriology, *eighth edition by Professor T. J. Mackie and Dr. J. E. McCartney, published by E. and S. Livingstone, Ltd, Edinburgh.*

Mannitol..Mauve	Salicin....Pink and White
Mannose..Black and Green	Sorbitol ..Black and Blue
Raffinose..Red and White	Starch ....Yellow and Mauve
Rhamnose Black and Pink	Trehalose Mauve and Green
Saccharose Blue	Xylose....Red and Green

Where colours are mentioned for which there is no cotton-wool, and where screw-cap bottles are used, the sides of the tube or the tops of the screw-caps may be "dabbed" with cellulose paints of the respective colours.

Culture media likely to be in regular use

Coloured beads are placed in the containers in which media are stored.

Medium	*Colour of bead*
Digest broth	Black
Nutrient agar (made from above)	Black
Infusion broth	Yellow
Nutrient agar (made from above)	Yellow
MacConkey's L.B A.	Red
Peptone water (no indicator)	White
Peptone water (with indicator)	Brown
MacConkey's fluid medium	
(single strength)	1 red spot (cellulose paint)
(double strength)	2 red spots
Sabourards medium	Light blue
Solutions	
Distilled water	White
N/saline	Dark blue
Dextrose in saline	Blue and green

Collection of Blood for Use in the Wassermann Reaction and Preparation of Serum media

Many laboratories have a standing arrangement with local slaughter-houses whereby supplies of blood and serum can be

obtained. It is advisable for a technician to make the actual collection at the time of killing and, if handled diplomatically, most slaughtermen will co-operate regarding any reasonable precautions to be taken against gross contamination of the blood.

Blood for use in the haemolytic systems of the Wassermann and similar reactions

250 ml. wide-mouth bottles, fitted with rubber bungs and containing two to three dozen small glass beads or short lengths of 5 mm. glass rod, are sterilized in the autoclave. Immediately bottles are half filled with blood at the slaughter-house they are shaken vigorously for at least five minutes (or until the beads cease to rattle, owing to being caught up in a fibrin mesh). When returned to the laboratory the bottles are placed in the refrigerator, well away from the cooling unit as actual freezing bursts the red-cells.

Blood for coagulated serum media

A large, lidded enamelled bucket is "burned out" with alcohol and the lid fixed firmly on. At the slaughter-house the bucket is three-quarters filled with blood which is allowed to clot. With a clean long-bladed knife the clot is cut into segments and the bucket allowed to stand in the cool (the slaughter-house authorities will usually allow the use of the cool-room for this) for twenty-four hours. The following day, sterile Winchester bottles containing about 100 ml. of chloroform, and a sterilized rubber syphon tube are taken, and the clear serum syphoned into the bottles. The serum is stored in a cool place and shaken occasionally to distribute the chloroform.

CHAPTER XX

BACTERIOLOGICAL FILTERS

THE objects for, and the methods of filtration in the bacteriological laboratory are multitudinous: from clarifying to sterilization of fluids and from segregation of species of bacteria to the extraction of toxins and other bacterial products.

The more common objects of filtration can be listed as follows:

(*a*) Removal of coarse particles from fluids.

(*b*) Prevention of air-borne organisms from entering cultures and sterile media, etc.

(*c*) Removal of mucoid material from specimens.

(*d*) Sterilization of fluids which would be damaged or destroyed by other methods of sterilization.

(*e*) Preparation of bacteria-free solutions of toxins.

(*f*) Separation of micro-organisms of varying sizes

(*g*) Measurement of ultra-microscopic organisms.

As the objects for filtration vary so do the methods employed. Items (*a*), (*b*) and (*c*) are dealt with under "Culture Media Preparation", page 145.

Chamberland filters

These filters are made from unglazed porcelain which consists of kaolin (hydrous aluminium silicate) and quartz sand heated to a temperature sufficiently low to avoid sintering (partial welding together of the particles). These filters, usually referred to as Chamberland "candles" because of their shape, are manufactured in a wide range of porosities, all of which are denoted by special marks on the candles.

L.1 (*most porous*). This is used for clarifying fluids only and is not intended as a bacterial filter.

L.1a (*coarse*). The maximum size of a particle likely to pass through this filter is 500μ, so that whilst most bacteria may be retained, the filter cannot be used for sterilization purposes as many micro-organisms are less than this in size.

L.2 (intermediate). The porosity of this filter is somewhat finer than that of the L.1a, but is not, nevertheless, sufficiently fine to exclude all bacteria.

L.3, L.4 and *L.5, etc.* These filters are of porosities fine enough for use in most bacterial filtrations.

Berkefeld filters (see fig. 35)

Originally of German manufacture, but now manufactured in this country, these filters are made from Kieselguhr (a fine white powder consisting of diatomaceous earth) and asbestos mixed together, shaped into cylinders and baked at a temperature of approximately 1,200° C. They are graded into three types which compare with the Chamberland filters :

"V" (Viel)—coarse—equal to Chamberland L.1a.
"N" (Normal)—medium—equal to Chamberland L.2.
"W" (Wenig)—fine—equal to Chamberland L.3.

Recent tests carried out on these filters have yielded the following result :

0·1 ml. of the filtrate from suspension of C. prodigiosum of 450,000 organisms per ml. produced no growth.

Mandler filters

American filters composed of diatomaceous earth, asbestos and plaster of paris in approximate proportions of 60–80 per cent. : 10–30 per cent. : 10–15 per cent. respectively, are also extensively used, and it is claimed that they are more robust for general laboratory purposes.

The porosity of these filters is gauged by the rate of flow of pure distilled water, kept at constant pressure, through them.

Plaster of paris

A "home-made" filter which has the advantage of cheapness and easy preparation is described in *Fundamental Principles of Bacteriology*, Salle, 1948 edition, page 187.

Sintered glass

Discs consisting of finely ground glass, heated to a temperature just sufficient to fuse the particles together without actually

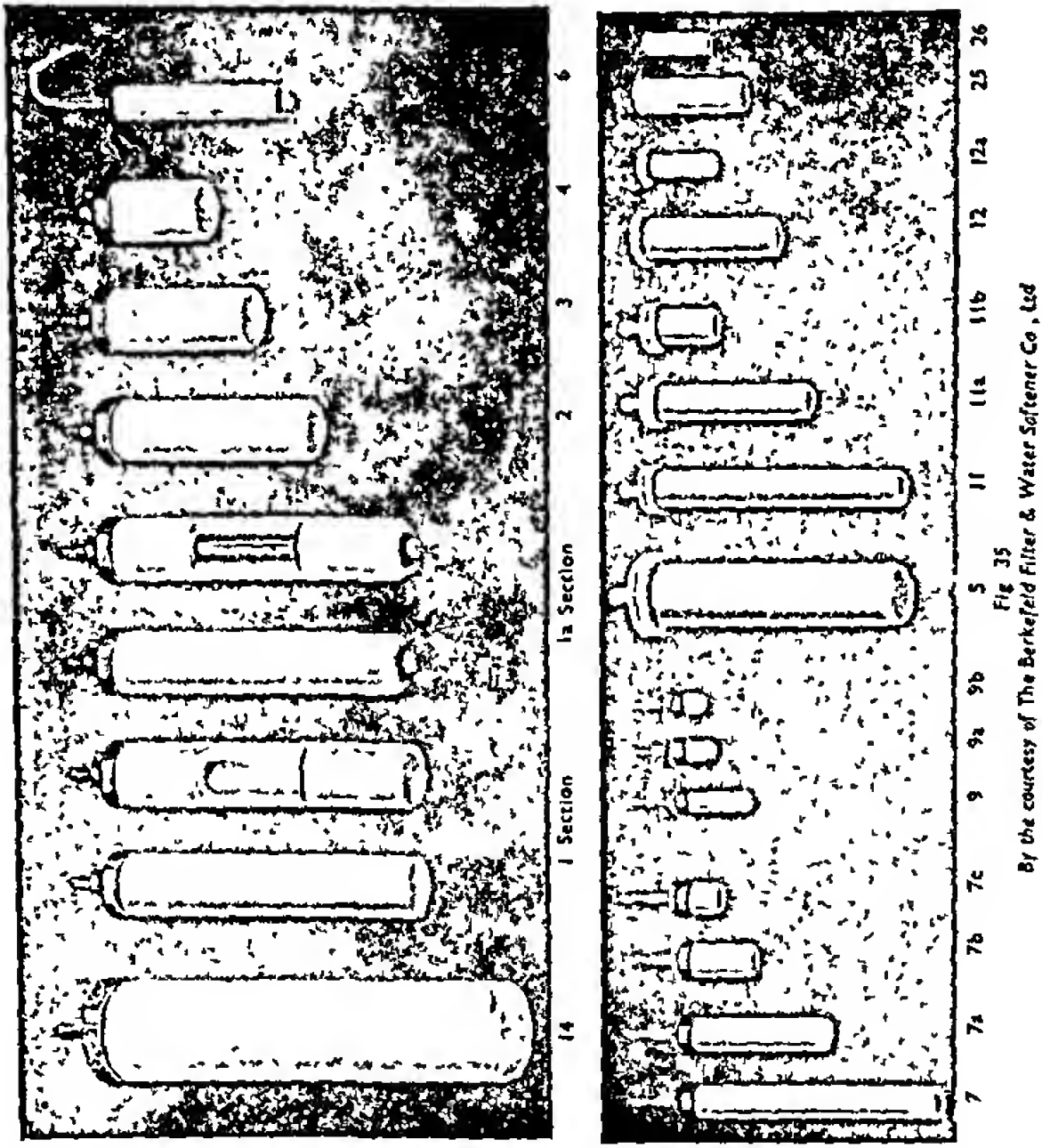


Fig 35
By the courtesy of The Berkefeld Filter & Water Softener Co., Ltd

melting them, are welded into glass funnels. These, like the previous filters, can be graded according to their porosity, and further porosity can be governed by the size of the glass particles employed in preparing the discs.

Collodion (Elford's "Gradacol")

Great accuracy in the size of the pores of these filters can be achieved in preparation and they are used to determine the size of many viruses. Nitro-cellulose (collodion) is

dissolved in acetone and diluted with a mixture of ethyl alcohol and ether with varying amounts of amyl alcohol added according to the porosity required. The mixture is poured into shallow dishes and allowed to evaporate for varying periods of time of from one to three hours, after which they are washed for a long period with distilled water. By varying the amounts and composition of the mixture and the conditions of evaporation, different porosities of from 3 to 10$M\mu$ or even less have been obtained.

Seitz filter

This apparatus consists of a metal funnel into which is fitted specially prepared asbestos pads (see Fig. 36).

Three types of pads are available :

(Sterimats)— *English*	(Seitz)— *Foreign*	
F.C.B.	K	*Coarse filter for* clarifying.
G.S.	EK	For routine bacterial filtrations.
S.B.	EK (special)	For very fine filtrations.

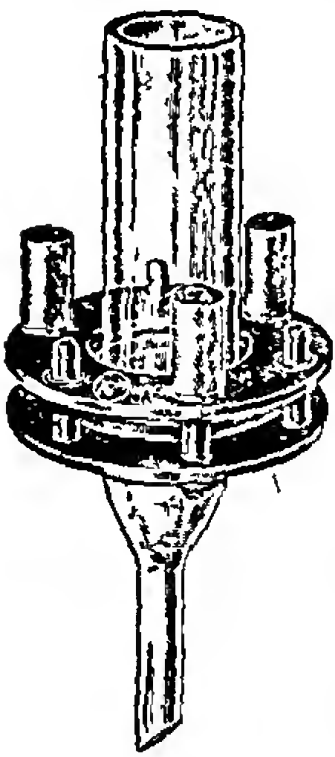


Fig 36.
English Counterpart of Seitz Filter
By the courtesy of Messrs A Gallenkamp Co Ltd

The pads are reasonably cheap and are discarded after use. The apparatus is particularly serviceable in the media-room for routine sterilization of such media as sugar media and those containing serum or similar fluids.

The "Technico" (English counterpart of the original Seitz filter) has the advantage of a resistance-glass interior which obviates the possible risk of bacteriostasis through oligodynamic action of traces of metals dissolved in fluids during filtration.

Electrical Charge of Filters

It must be carefully borne in mind that fluids may suffer partial or even complete loss of their active properties in course of filtration. This is particularly important when

dealing with solutions of toxins, antitoxins, etc., of which the titre must be established. Porcelain, asbestos and other materials used in the manufacture of these types of filters consist of metal silicates.

Positive ions (cations) of some of the metals are more soluble than the silicate negative ions (anions) and, during filtration, tend to pass into solution. During filtration reaction between the "positive" and "negative" ions of the material being filtered and the substance of the filter may occur and result in the retention in the filter of ions of the material which have reacted with the oppositely charged ions of the substance of the filter. And, of course, further changes may occur in the material due to similar reactions resulting in certain soluble substances passing out of the filter with the material being filtered. This can be easily demonstrated by pouring a weak solution of methylene blue into an ordinary porcelain filter; the filtrate will be much weaker or even colourless.

Pressures for Filtration

"Negative" (or vacuum) pressure is the best for filtrations Filtration should be commenced with the minimum amount of pressure, steadily increasing as the filtration proceeds The maximum pressure should not exceed 200 mm. of mercury. Prolonged filtration, particularly if dealing with long flexuous bacteria may result in small numbers of bacteria passing, lengthwise, through the pores of the filter. Too great pressure may result in clogging of the surface pores of the filter.

Fig. 37

Screw-cap attachment for bottles

Special screw-cap attachments for filtering direct into storage bottles may be obtained commercially. The apparatus (Fig. 37) is self-explanatory. Care must be taken to avoid contaminating the inside surface of the apparatus and the necks of the bottles during fitting and use.

Filtration Systems

Various forms of apparatus for sterilizing by filtration are illustrated below.

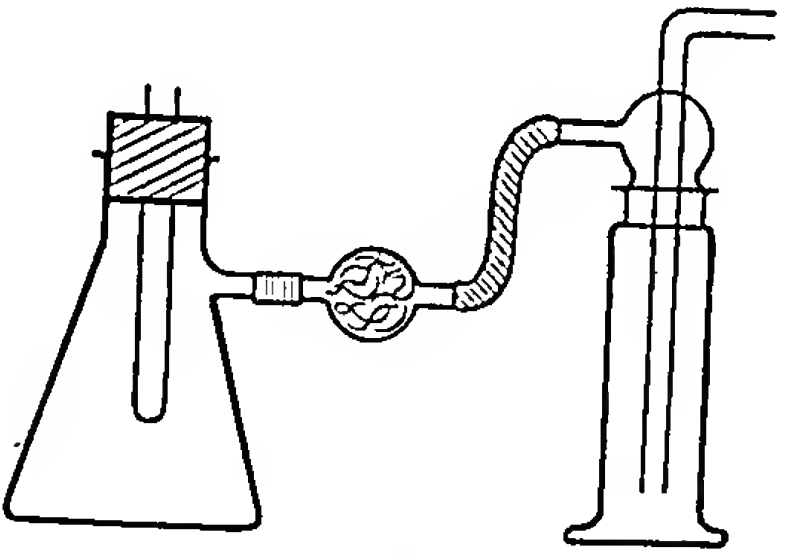


Fig. 38.

Fig. 38. A simple system consisting of a filter-candle fitted into a Buchner flask with a rubber bung. The flask is connected through a cotton-wool air-filter and Dreschel bottle (acting as a trap for any possible back-flow of water from the filter-pump).

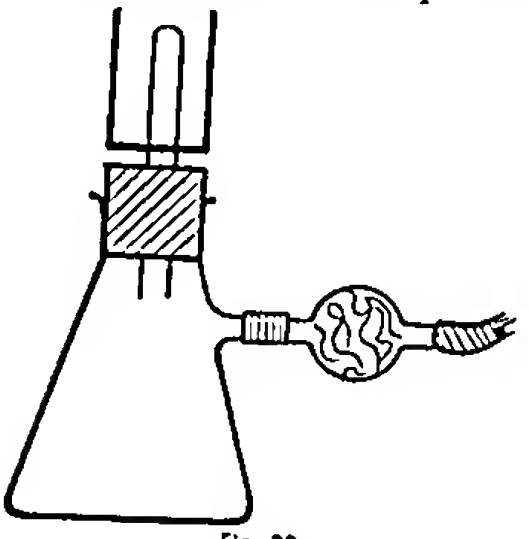


Fig. 39

Fig. 39. A similar system with a filter candle and glass mantle. This is the more efficient method when filtering comparatively small quantities and, which is important, the candle is more easily cleaned subsequently as the residue adheres to the *outer* surface of the filter and can be readily removed.

Fig. 40. A filtration system which comprises a measuring device for tubing or ampouling small measured quantities of sterile fluid. The manometer is inserted in the suction system as a control of the pressure employed, whilst the " trap " bottle is fitted with a glass tap for releasing pressure

whilst ampouling. The graduated tube is fitted with a hooded nozzle to reduce the risk of contamination whilst ampouling.

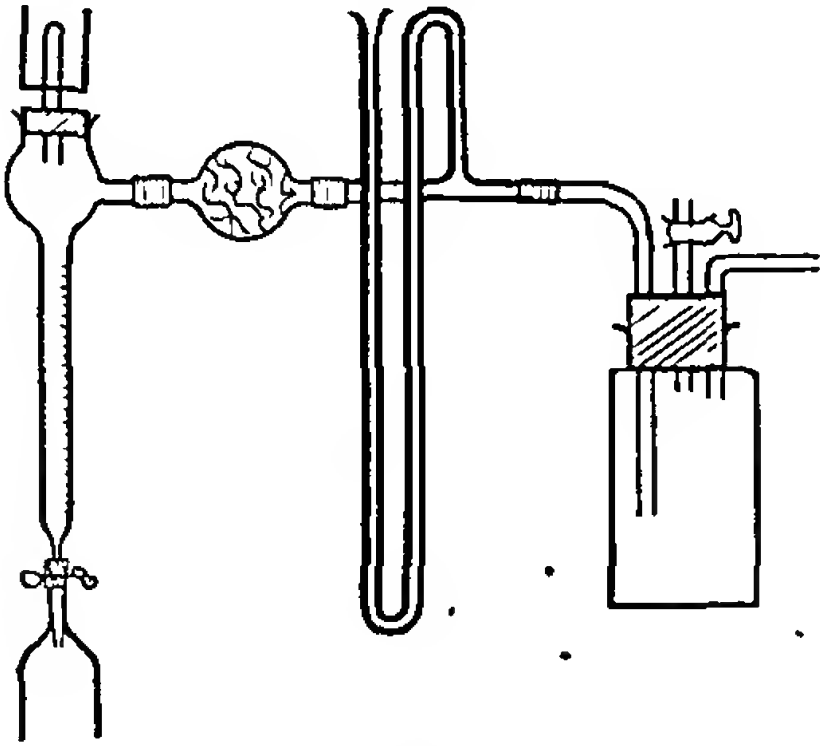


Fig 40

Automatic pipettes of various capacities are available commercially, and these may be introduced in place of the hooded pipette and graduated tube where constant volumes are to be ampouled.

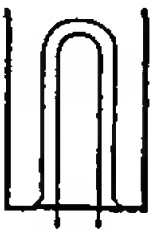


Fig 41

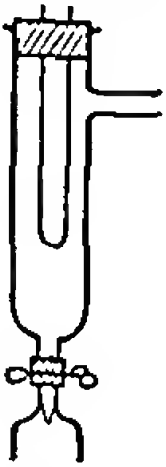


Fig 42

Fig. 41. Where very small quantities of fluids are to be filtered a small test-tube inverted over the candle will obviate wastage.

Fig. 42. A simple device for ampouling approximate quantities.

Cleaning bacteriological filters

Following sterilization after use, Chamberland, Berkefeld and Mandler filters may be cleaned by steeping them in distilled water and then drawing distilled water through them in the opposite direction to that in which the material has been filtered, by means of the vacuum pump. This will dislodge much of the material which may be clogging the surface pores of the filter. They can then be scrubbed with a stiff nail-brush, followed by boiling in distilled water for twenty to thirty minutes. Should a filter be badly clogged with organic matter it may be slowly heated to redness and allowed to cool over a long period of time by burying it in asbestos fibre.

Testing filters

To test a filter a current of air is pumped through the filter, whilst it is completely immersed in distilled water. Any cracks or flaws will be revealed by large bubbles appearing at the damaged part, whilst with undamaged filters very fine comparatively even-sized bubbles will appear over the entire surface of the filter.

CHAPTER XXI

SECTIONS

THE subject of section preparation, cutting and staining really comes in the province of Histological and Pathological technique, but it is necessary on occasions for bacteriological investigations to be carried out on tissues

In addition to culturing the micro-organisms present, it is sometimes desirable to demonstrate them *in situ* or to note their effects upon the tissues, etc.

For practically all bacteriological work, fixation and dehydration, followed by embedding in paraffin wax suffices.

For more complete instruction on section cutting and staining the reader is referred to *Practical Section Cutting and Staining* by E. C. Clayden (Churchill).

Selection of material

Except where required as museum exhibits, large masses of tissue should not be placed in the fixing solutions and left for lengthy periods for the solution to penetrate. Small pieces should be selected and immersed in the chosen fixative immediately upon arrival at the laboratory.

Fixation

According to the investigation on hand, small pieces of tissue are immersed in the fixative, care being taken to adhere to the technique recommended for the particular fixative employed. (See " Fixatives ", page 262.)

Washing

A gentle stream of water is better than frequent changes to fresh water.

Dehydration

Convection currents and inter-action between fluids of different specific gravities and composition are to the delicate tissue cells rather like a raging torrent to a flimsy structure

across an otherwise peaceful stream. They just will not "stand up to it" and become distorted and damaged beyond recognition. The damage will not become evident until the final sections are under the microscope, and then it is too late, the damage cannot be remedied. The process of dehydration should be a gradual one. Transference of the tissue through a series of gradually increasing strengths of alcohol up to "absolute" is essential for good results.

Clearing

From the alcohol the tissue is placed in a "clearing" fluid such as xylol, chloroform or benzene where, if it has been completely dehydrated, it becomes semi-transparent. Incomplete dehydration will be readily revealed at this stage by the appearance of unchanged areas in the tissue.

Impregnation with wax

The more rapidly this is performed the better for the sections. Overheating or too lengthy a stay in the paraffin bath will result in damage to the tissue, and to the microtome knife when cutting, as tissues shrink and become hard and brittle if so treated. Vacuum-embedding baths have much to recommend them as the process is speeded up without detriment to the tissues. At least one change of wax should be made during impregnation to avoid traces of the clearing fluid being taken over to the wax in which the tissue is blocked. Wax of melting point 60° C. is recommended as it gives a reasonably firm substance for cutting both in winter and summer.

Blocking

A separate vessel containing wax for preparing blocks should be kept in the embedding bath. L-shaped moulds or small paper cups may be used in which to "block" tissues. The mould or cup is filled with molten wax and, using warmed forceps, the tissue placed in the molten wax, care being taken

[illegible] this method

[illegible] ig

in the wax as with the latter the surface of the tissue solidifies,

causing a fine gap between the tissue and the bulk of the wax block).

When hardened, the wax block is trimmed with an old scalpel or penknife leaving a narrow margin of wax round the tissue The block is then fixed to the block-holder of the microtome by warming the blade of a metal spatula and "sandwiching" the blade between the top of the holder and the under-surface of the block until the wax on both begins to melt, when the blade is withdrawn leaving the block and holder firmly pressed together until the wax hardens. The holder is next fitted to the rocker-arm of the microtome and adjusted until the surface of the wax block is directly over the knife-edge.

Cutting

A few movements of the rocker-arm will pare away the film of wax, leaving the surface of the tissue exposed to be cut into sections as desired.

Too wide a range of microtomes are in use in various laboratories and for different purposes, to be included here. The Cambridge "Rocker" type serves for most bacteriological investigations and we will confine ourselves to this particular type of instrument. It consists of a rocker-arm to which is attached a "block-holder" and which rocks up and down across the edge of a knife fitted to the instrument. A device is incorporated which propels the rocker-arm forward towards the knife each time the arm is moved The thickness of the section cut can be controlled by regulating the forward travel of the rocker-arm. The instrument should be kept scrupulously clean and lubricated with thin oil (typewriter oil is suitable) It should be kept covered when not in use.

Mounting sections

As sections are cut they are lifted from the knife blade with a fine camel-hair brush and transferred to the surface of some clean water kept at a temperature just below the melting point of the wax used, where they will straighten out and lie flat. A cover-slip, held in forceps, or a slide is dipped

into the water, brought near a section until one edge of the section touches it. The cover-slip/slide is then gently withdrawn from the water, perpendicularly, when the section will adhere to it, being lifted clear of the water and lying flat on the cover-slip/slide. Before any further steps can be taken the section must be allowed to dry thoroughly either in air or in the incubator.

Preparing sections for staining

Xylol, chloroform or benzene is dropped on the section until all trace of wax is removed. (IMPORTANT.—From now on the section must not be allowed to dry otherwise it will curl or peel off and be lost in subsequent stages of the process.) The section is next passed back through the various dilutions of alcohol to water, after which it may be stained by the particular method chosen.

Final mounting

Following upon the staining operations the section is again dehydrated and cleared and, before the clearing fluid can dry out, a small drop of canada balsam or Euparol (recommended) is placed on the section and (*a*) the cover-slip pressed gently on to a slide so that the section is embedded in the mounting fluid between the slip and the slide or (*b*) a cover-slip pressed gently on to the slide preparation.

A recent modification in section technique is the introduction of (*a*) Cellosolve (ethylene glycol monoethyl ether) which is miscible with water, alcohol and xylol and which, introduced into the technique between the 75 per cent alcohol and xylol, ensures more efficient dehydration.

(*b*) Dioxan (diethylene dioxide) which is miscible with water, xylol and paraffin wax. This may be used where urgency demands less exacting technique—small pieces of tissue, after washing, are immersed in dioxan for 2–3 hours, transferred to 50:50 dioxan and paraffin wax at a temperature just above the melting point of the wax for 1 hour, and then into pure wax for 1 hour. After this the tissue may be blocked.

The miscibility of Dioxan with water, xylol and wax opens up a wide field for research in staining techniques which may dispense with much of the hydration and dehydration of sections.

Labelling Tissues whilst Processing

A series of small standard-sized wide-necked bottles with corks to fit are set up as shown in Fig. 43. A number of corks of similar size to those in the bottles are fitted with lengths of stainless steel wire twisted at each end to form clips.

When the piece of tissue is selected it is lightly fixed into the lower clip of a cork and a label fastened to the upper clip.

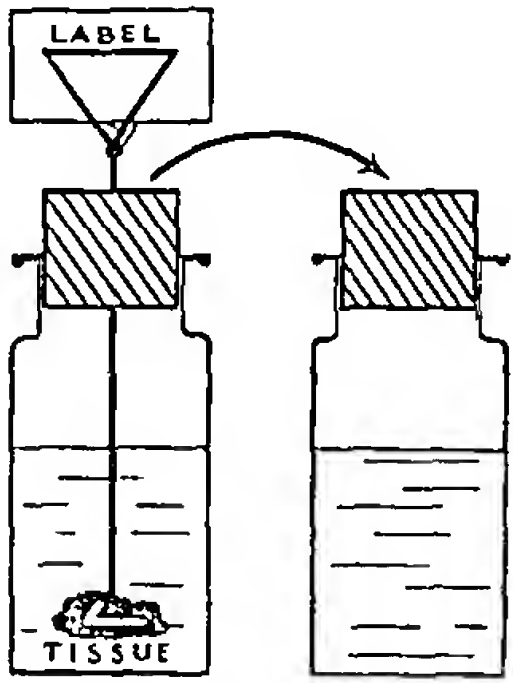


Fig 43

This cork can then be transferred along the row of bottles, replacing corks as it moves along. Further, a number of tissues can follow closely upon each other without risk of becoming wrongly-labelled. At the wax-embedding stage small flat-bottomed specimen tubes may be used for individual specimens and the labels attached with rubber bands.

CHAPTER XXII

ROUTINE BACTERIOLOGICAL EXAMINATION OF WATER

THE routine bacteriological examination of water is carried out to establish :

(*a*) Whether the water is fit for human consumption ?
(*b*) Whether the water is fit for general use (ablution, etc.) ?
(*c*) Whether the water is likely to cause trouble in the mains?
(*d*) The possible bacterial cause of stagnation, odour or taste in water supplies.
(*e*) Possible presence of known pathogenic organisms.

The technique involves methods for :

(*a*) Enumeration of viable bacteria.
(*b*) Quantitative Coliform test.
(*c*) Special examinations for presence of specific bacteria-pathogens or organisms responsible for specific abnormalities in the water supply.

Collection of samples

Various outfits are obtainable commercially for this purpose but ground-glass stoppered bottles of approximately 230 ml. capacity, wrapped in thin kraft-paper and autoclaved are quite suitable. Metal containers consisting of an inner chamber in which the bottle of sample is placed, fitted into a larger container in which ice and sawdust are packed in the event of possible delay in the sample reaching the laboratory, should be provided. The bottle should be weighted so that it sinks readily if lowered into a reservoir, river, well, etc., and should have a long length of string attached to both the bottle and the stopper.

If collecting a sample from a tap, the mouths of both the tap and the bottle should be flamed and the tap allowed to run for at least five minutes before the sample is collected. This precaution obviates the possibility of water that has been standing in comparatively warm upper sections of piping for some while being taken, it is a representative sample of water from the mains that is required in this instance. If collecting water from a well, reservoir, river, etc., the weighted

bottle should be lowered well below the surface of the water and opened by a jerk of the string attached to the bottle and the stopper. The bottle is then hauled to the surface where the upward current of water which will come with it will temporarily wash away surface contaminations whilst the bottle is lifted clear. The stopper is then immediately replaced, pressed securely in and fastened by means of the string.

Enumeration of viable bacteria

Apparatus required :

Sterile 1 ml. pipettes and 10 ml. pipettes.
Sterile petri-dishes.
Tubes of nutrient agar (pH 7·2) containing 10 ml.
Bottles containing 90 ml. sterile distilled water.

Technique. If the water sample is suspected of heavy contamination series dilutions are made by adding 10 ml. of the sample to 90 ml. sterile distilled water, mixing and transferring 10 ml. of the mixture to a further 90 ml. of sterile water and so on, making dilutions of 1:10, 1:100, 1:1,000, etc.

Similar technique to that for the neat sample is employed in respect of each dilution used, so that it is necessary only to describe that for the undiluted sample The agar is melted and cooled to 50° C. 1 ml quantities of the sample are measured into each of two petri-dishes, 10 ml. of agar added to each dish and the contents thoroughly mixed by rotating the dishes whilst holding them in a horizontal position and taking great care that the agar does not spill over the edges. The plates are left on the bench, with the lids slightly tilted until the medium has solidified and the surface is dry. The plates are then labelled and one incubated at 37° C. for twenty-four hours and the other at room temperature for forty-eight hours. Each colony which develops represents one viable bacterium for the purpose of counting Counting should be done with a low-power hand-lens (×5) An enumerating disc, such as Pake's (Fig. 44), is preferable to dividing the plate into approximately equal segments with a grease-pencil. The average count for municipal water supplies, after filtration is 100 per ml A count of 1,000 per ml. or over is viewed with suspicion In "normal" waters the colonies growing

at room-temperature should exceed those at 37° C. for reasons which are obvious !

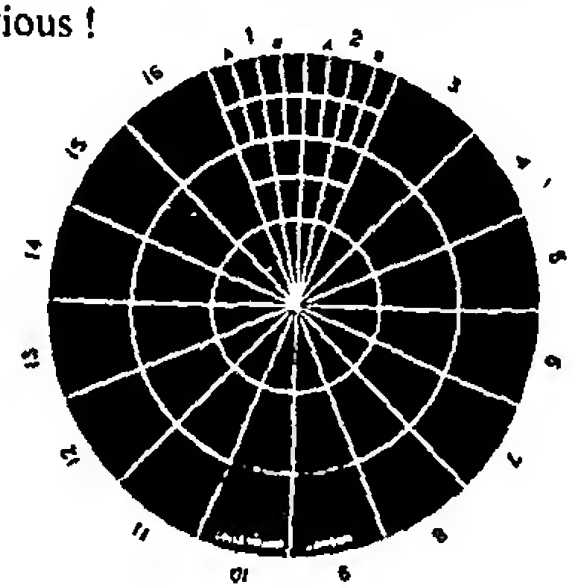


Fig. 44
PAKE'S DISC.

Quantitative coliform test

The number, if any, of coliform bacilli in a sample of water can be expressed either as :

(*a*) The number of organisms in a given volume ; or

(*b*) The smallest volume of water to contain coliform bacilli.

The culture medium used is MacConkey's fluid medium which contains bile-salts and is designed to restrict the growth of organisms other than those which normally inhabit the lower colon of man and animals (see " Culture Media ", page 154).

For the test the following are required :

1 flask (or screw-cap bottle) containing 50 ml. MacConkey's medium—double strength (D.S.).

5 tubes (or small bottles) containing 10 ml. MacConkey's medium—double strength.

5 tubes (or small bottles) containing 10 ml. MacConkey's medium—single strength (S.S.).

And, if the water is suspected of heavy contamination, a further 5 tubes of 10 ml.—single strength.

Using sterile pipettes quantities of the sample are measured into the flasks and tubes as follows :

1 50-ml. flask : 50-ml. sample.
5 10-ml. tubes (D.S.) : 10-ml. sample into each.
5 10-ml. tubes (S.S.) : 1-ml. sample into each.

If required 5 10-ml. tubes (S.S.) : 0·1-ml. sample into each.

All flasks and tubes are clearly labelled as to their contents and incubated at 37° C. for forty-eight hours

McCrady's "Probability Table" of Coliforms in Water Samples.

Volume of water	50 ml.	10 ml.	1 ml.	Probable number of coliforms in 100 ml. water
Number of samples of each quantity	1	5	5	
	0	0	0	0
	0	0	1	1
	0	0	2	2
	0	1	0	1
	0	1	1	2
	0	1	2	3
	0	2	0	2
	0	2	1	3
	0	2	2	4
	0	3	0	3
	0	3	1	5
	0	4	0	5
	1	0	0	1
	1	0	1	3
	1	0	2	4
	1	0	3	6
Number of tubes showing positive reaction.	1	1	0	3
	1	1	1	5
	1	1	2	7
	1	1	3	9
(Acid with production of gas.)	1	2	0	5
	1	2	1	7
	1	2	2	10
	1	2	3	12
	1	3	0	8
	1	3	1	11
	1	3	2	14
	1	3	3	18
	1	3	4	20
	1	4	0	13
	1	4	1	17
	1	4	2	20
	1	4	3	30
	1	4	4	35
	1	4	5	40
	1	5	0	25
	1	5	1	35
	1	5	2	50
	1	5	3	90
	1	5	4	160
	1	5	5	180 +

Under the old system the smallest amount of sample to give a positive reaction (production of acid and gas) was reported to be the quantity of sample to contain E. coli.

i.e. "E. coli present in, say, three 1-ml. tubes of sample, absent from 10 ml."

The modern method is the use of "McCrady's Probability Tables", see page 197.

Confirmatory tests for coliform organisms

Any coliform organisms present must be investigated further, and to do this pure cultures of the organisms must be obtained by plating out sample "Positive" tubes on to lactose-bile-salt-agar plates, picking off what appear to be typical colonies and sub-culturing these on to agar slopes and into peptone water tubes.

The "Coliform" family of organisms comprise a heterogeneous group of organisms all very similar in morphology and in many of their biochemical reactions. Some of them, however, exist outside the mammalian intestine and do not, necessarily, indicate faecal pollution. Tests to distinguish between "Typical" and "Atypical" coliforms must therefore be carried out. These tests include :

Indole reaction.
Sugar-fermentation reactions.
Voges Proskauer reaction.
Methyl red reaction.
Citrate utilization.
Gelatin liquifaction.

The common excretal type of coliform organism gives the following reactions :

Lactose	Inositol	Indole	V. Proskauer	Meth. Red	Citrate utilization	Gelatin liquifaction
A G	N C	Pos.	Neg.	Pos.	Neg.	N L

A G=Acid and gas. N C=No change. Pos=Positive. Neg.=Negative. N L=No liquifaction.

Test for Indole

The organism is grown in peptone water medium for forty-eight hours at 37° C. and 2-3 ml. of the culture mixed in a

test-tube with a small quantity of Erlich's Rosindole reagent :

P-dimethyl-amino-benzaldehyde	4 grms.
Absolute alcohol	380 ml.
Pure hydrochloric acid	80 ml.

A rose-colour denotes a positive reaction (presence of indol). The addition of a saturated solution of potassium persulphate is said to speed up the reaction. The indole can be extracted from the culture with amyl alcohol or ether by shaking a small quantity of either with the culture and decanting off. The concentrated solution of indole in the amyl alcohol (or ether) is then added to the rosindole reagent.

Sugar reactions

The organism is inoculated into the following sugars and incubated for at least forty-eight hours.

	Lactose	Glucose	Dulcitol	Saccharose
Typical coli	+	+	+	−
Atypical coli	+	+	+	±

\+ Acid and gas
− Acid only.
± Reaction varies with strains of the organism.

Voges Proskauer reaction

Method 1 2 ml. of 10 per cent. NaOH solution is added to a forty-eight-hour culture of the organism in 10 ml. of glucose broth and allowed to stand at room-temperature for several hours. An "eosin-pink" colour denotes a positive reaction.

Method 2 A trace of creatin in 5 ml of 40 per cent. NaOH is added to the glucose-broth culture. The same colour tint denotes a positive reaction and develops much more rapidly than in Method 1.

Methyl red reaction

This test depends upon the pH of the culture of organisms in a standard glucose-broth medium after seventy-two hours incubation at 37° C.

To 10 ml. of a seventy-two-hour culture of the organism two drops of methyl red solution is added. A red coloration denotes a positive reaction (pH 4·5 or less).

Methyl red solution:

Methyl red	0·1 grm.
Absolute alcohol	300 ml.
Distilled water up to	500 ml.

For the standard glucose-broth, see "Culture Media", page 160.

Citrate utilization

The principle underlying this test is that non-faecal coliform types are able to synthesize metabolites from inorganic substances (Autotrophic), obtaining nitrogen from non-protein matter and carbon from the citrate (as against obtaining organic carbon from carbohydrates as do the coliform organisms which inhabit the intestine). For the medium used, see "Culture Media", page 159.

The organism under investigation is subcultured into tubes of the medium and incubated.

Growth denotes—NON-FAECAL types
No growth denotes—Faecal types.

Gelatin liquifaction

For the medium used, see "Culture Media", page 153.

Stab-cultures are made into tubes of the nutrient gelatin, and these are incubated at room-temperature (or in a 22° C. incubator). Whilst non-faecal types of coliform organisms vary regarding liquifaction of gelatin, faecal types never do so.

The Ministry of Health recommends that "piped water" (tested at entry) should be classified as follows:

Class 1. Highly satisfactory, less than 1 coliform per 100 ml. water.
Class 2. Satisfactory, less than 2 coliforms per 100 ml. water.
Class 3. Suspicious, 3 to 10 coliforms per 100 ml. water.
Class 4. Unsatisfactory, over 10 coliforms per 100 ml. water.

Eijkman's reaction

This reaction is based upon the fact that coliform organisms grown in MacConkey's fluid medium at 44° C. give the following results:

Faecal types	" Acid and gas ".
Non-faecal types	" Acid only " or " No growth ".

The reaction has recently been included in the routine procedure for bacteriological investigation of water supplies by many laboratories. Two methods have been devised. (*a*) Duplicate sets of MacConkey's medium are inoculated (as on page 196). Of these, one set is incubated at 37° C. and the other at 44° C in a water-bath for forty-eight hours.

(*b*) One set of MacConkey's medium is incubated at 37° C. *in the usual manner and all bottles and tubes showing* " acid and gas " are subcultured into 10 ml. single-strength tubes of the same medium and immersed in the 44° C. water-bath for forty-eight hours.

Tubes showing " acid and gas " indicate the presence of faecal-type coliforms in the tubes from which the subcultivation was made.

Note. The following points are important.

1. The 44° C. water-bath must not deviate more than ±0·5° C
2. The bath must have a well-fitting lid.
3 Bottles and tubes must be immersed in the water to at least the depth of the medium.

Complete closure of the bath and immersion of the bottles and tubes avoids convection currents within the medium which would create a variance in temperature beyond the scope of the thermostat to control.

Test for faecal streptococci

The faecal streptococcus is a common inhabitant of the intestine and its presence in water indicates faecal pollution. The test need only be carried out if pollution is suspected and no typical coliform organisms are demonstrated. Tubes

from the "coliform test" series which show acid without gas production are selected.

Method 1. This method is based upon the resistance to heat of the faecal streptococcus. To 1 ml. of the selected tube or tubes 9 ml. of sterile distilled water is added and the tubes heated in a water bath at 60° C. for fifteen minutes. Samples from each tube selected are then plated out on lactose-bile-salt agar and incubated for forty-eight hours. Tiny red colonies appearing on the plates indicate the presence of faecal streptococci.

Method 2. Selected tubes from the "coliform" series are plated out on lactose-tellurite agar (see "Culture Media", page 157). Colonies of faecal streptococci appear as small blue-black discs.

Test for clostridium welchii

The clostridia are saprophytic organisms commonly occurring in farmyard manure and other excretal matter and the presence of these organisms indicates possible faecal contamination. One of the clostridia family which can prove extremely harmful is the C. welchii (the causative organism of "gas-gangrene"). 50 ml. of the sample of water is added to 100 ml. of sterile milk in a screw-cap bottle or plugged flask. The mixture is heated to 80° C. for fifteen minutes (to kill off the vegetative bacteria and leave any spores which may be present viable) and a layer of liquid paraffin (or molten petroleum jelly) poured over the surface of the mixture to preserve anaerobiosis. The bottle or flask is then incubated at 37° C. for forty-eight hours. Clotting of the milk coupled with the presence of wide gas-fissures throughout the clot (stormy-clot reaction) together with a distinct odour of butyric acid indicates the presence of C. welchii.

Sewage and Sewage Effluents

As crude sewage may contain as many as from 1 to 100 million organisms per ml. the technique detailed above may be modified by making suitable dilutions of the samples. The isolation and investigation of possible pathogenic organisms may be carried out as for stools (see page 214).

Where the presence of typhoid and paratyphoid organisms is suspected in water supplies 900 ml. of the sample is added to 100 ml. of a solution consisting of :

Peptone	10 grams.
Sodium chloride	5 „
Brilliant green solution (1:1,000)	5 ml.
Water	100 „

and incubated at 37° C. Subcultures are made on to selective media at twenty-four and forty-eight-hour intervals.

CHAPTER XXIII

ROUTINE BACTERIOLOGICAL EXAMINATION OF MILK

As for water samples, the object for routine bacteriological examination of milk is to ensure that it is fit for human consumption and that a standard of quality is maintained. The *Ministry of Health* " *Milk (Special Designations) Regulations*, 1936-46 " classify milk supplies as :

1. *Tuberculin Tested.* All animals from which this grade of milk is taken are subjected to special tuberculin tests at regular intervals and animals satisfying the requirements of the test are certified, suitably marked and registered.

Samples of this milk are required to:

(*a*) Fail to decolorize a standard amount of methylene blue in a given length of time: 1st May to 31st October, 4½ hours; 1st November to 30th April, 5½ hours.

(*b*) Contain no coliform bacillus in one hundredth of a millilitre.

1a. *Tuberculin Tested* (*Pasteurized*). In addition to tuberculin testing the animals, the milk is pasteurized.

Samples of this grade of milk are required to:

(*a*) Fail to decolorize a standard amount of methylene blue in 30 minutes.

(*b*) Show a reading of not more than 2·3 Lovibond units when subjected to a standard Phosphatase test.

(*c*) Contain no coliform bacillus in one hundredth of a millilitre.

2. *Accredited.* Animals from which this grade of milk is taken are subjected to examination by a veterinary surgeon once in every [illegible] and all cow-pats showing evidence of disease likely to affect [illegible] included in the bulk of the [illegible]

Samples of this milk are required to:

(*a*) Fail to decolorize a standard amount of methylene blue within a given length of time.

(*b*) Contain no coliform bacillus in one hundredth of a millilitre.

3. *Pasteurized.* All milk so designated is pasteurized (retained at 145° to 150° F. for at least 30 minutes and immediately cooled to not more than 55° F.).

Samples of this milk are required to contain not more than 100,000 bacteria per millilitre.

So that the examination of a sample of milk may involve such of the following tests as apply according to the designation of the milk.

1. Estimation of the total number of bacteria by "plate-count".
2. Estimation of the total number of bacteria by methylene blue test.
3. Estimation of the total number of coliform bacilli present.
4. A check upon the effect of pasteurization (phosphatase test).

Estimation of number of viable bacteria (for pasteurized and undesignated milk)

For each test the following apparatus is set out (see Fig. 45).

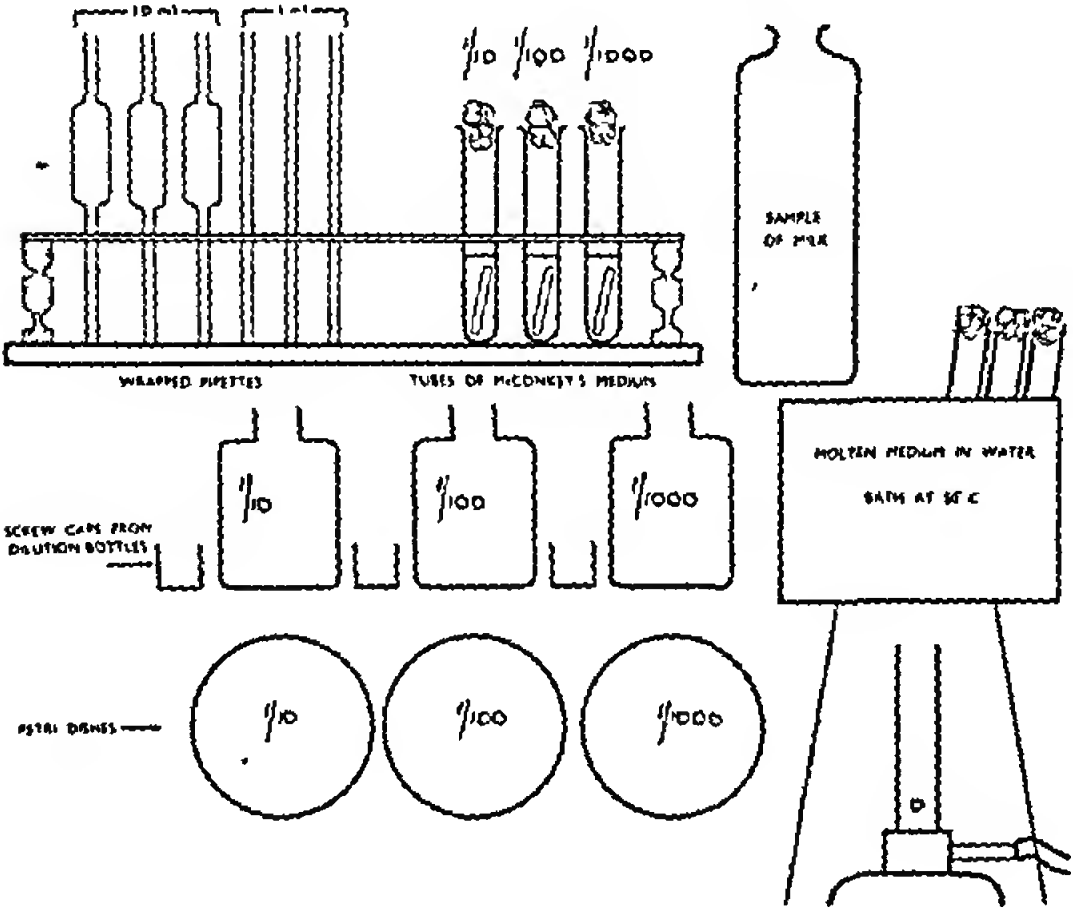


Fig 45.

(*a*) three screw-cap bottles (100 ml.) each containing 90 ml. sterile water.

(*b*) three tubes of single-strength MacConkey's fluid medium.

(*c*) three 10 cm. diameter petri-dishes (sterile).

(*d*) three sterile 10 ml. pipettes.

(*e*) Three sterile 1 ml. pipettes.

(*f*) Three 10 ml. tubes of special medium, melted and cooled to 50° C. (see " Culture Media ", page 153).

Technique. The sample of milk is mixed thoroughly by inverting the bottle at least twelve times. The neck of the bottle is flamed, the stopper removed, the bottle stood down on the bench and the bunsen flame again passed round the rim of the bottle. The screw-caps are removed from the dilution bottles, using the same sterile precautions as before, and the caps are placed, inverted, on the bench in front of the respective bottles.

Using a sterile 10-ml. pipette, 10 ml. of the sample is transferred to the first bottle and the pipette rinsed out several times by drawing up the mixture and allowing it to drain back into the bottle (do not blow it out). The cap of the bottle is replaced and the contents shaken. Using a fresh pipette, 10 ml of this mixture is transferred to the second bottle and the rinsing and shaking repeated. 10 ml. of the second dilution is then transferred to the third bottle, again using a fresh pipette and rinsing and shaking as before. With a 1-ml. pipette 1 ml. of the 1:10 dilution is transferred to the corresponding petri-dish and a further 1 ml. to the tube of MacConkey's fluid medium labelled 1:10. Using a fresh pipette each time similar quantities are transferred from the remaining two bottles to their corresponding petri-dishes and tubes of MacConkey's fluid medium. One 10-ml. tube of the special medium is added to each petri-dish and the contents thoroughly mixed by rotating the dish, keeping it perfectly horizontal and avoiding splashing the contents over the side of the dish. Tilt the lids of the dishes slightly until the medium is solidified and the surface quite dry and then place the plates and the tubes of MacConkey's medium in the 37° C. incubator. The plates are incubated for twenty-four hours, the MacConkey's medium for forty-eight hours. Each colony developing in the plates represents one viable bacterium.

Under the English regulations, plates showing over 500

colonies are disregarded unless the next highest dilution shows less than 30 colonies.

Eg:

1 ml of 1:1,000 dilution of milk showed 5 colonies.
1 ml. of 1·100 „ „ „ 28 „
1 ml. of 1 10 „ „ „ 520 „
Then the 1·10 dilution is considered, but if.
1 ml. of 1.1,000 dilution of milk showed 5 colonies
1 ml. of 1 100 „ „ „ 48 „
1 ml. of 1:10 „ „ „ 620 „
Then the 1/10 dilution is not taken into account.

Under the Scottish regulations a mean average of all plates is taken.

Methylene Blue Test

Sampling

Except where a sample consists of an unopened bottle or carton, the milk to be sampled must be well mixed and the sample must be collected under aseptic precautions in a sterile bottle.

Reagents and apparatus

(*a*) *Methylene blue.* Tablets manufactured under arrangements made by the Minister of Health must be used. Add one tablet to 200 ml. of cold sterile glass-distilled water in a sterile flask and shake until the tablet is completely dissolved. After the tablet has dissolved, make up the solution to 800 ml. with cold sterile glass-distilled water and store in a stoppered flask in a cool, dark place. The solution must be stored in the dark and not kept for longer than two months. When in use it must at no time be exposed to sunlight.

(*b*) *Test-tubes.* Test-tubes conforming to the British Standard Specification 152/16, nominal 6 in. × $\frac{5}{8}$ in., having an internal diameter of 13·5 ± 0·5 mm. and an etched mark indicating 10 ml., must be used. Test-tubes must be plugged with cotton wool, covered with closely-fitting aluminium caps, or otherwise stoppered so as to avoid contamination.

(*c*) *Rubber stoppers.* These must be sterilized before use.

(*d*) *Pipettes.* 1 ml. straight-sided blow-out delivery pipettes must be used for measuring the methylene blue solution. The pipettes must be plugged with cotton wool at the upper end, and be sterilized.

Method of carrying out the test

Thoroughly mix the sample of milk by inverting and shaking the sample bottle and pour the milk into a sterile test-tube up to the 10 ml. mark, leaving one side of the interior unwetted with milk. Add 1 ml. of methylene blue solution without letting the pipette come into contact with the milk in the tube or with the wetted side of the interior of the tube. After the lapse of three seconds, blow out the solution remaining in the tip of the pipette. Close the tube with a sterile rubber stopper taking aseptic precautions. Invert the tube slowly twice, so that the whole column of contained air rises above the level of the milk, and place within five minutes in a water-bath. The water in the bath must be kept above the level of the milk in the test-tubes, and its temperature, which must be between 37° C. and 38° C., maintained as nearly uniform as possible by means of a reliable automatic thermo-regulator. The interior of the bath must be kept completely dark.

To show when decolorization is complete a control tube must be used with each batch for comparison with the experimental tubes. The control tube is prepared by immersing in boiling water for three minutes a stoppered test-tube containing 10 ml. of mixed milk from a number of samples + 1 ml. of tap water. The milk used for the control tubes must be approximately of the same fat-content and colour as that being tested.

The milk is to be regarded as decolorized when the whole column of milk is completely decolorized or is completely decolorized up to within 5 mm. of the surface. A trace of colour at the bottom of the tube which does not extend upwards for more than 5 mm. may be ignored.

Precautions

The methylene blue solution when not in use must be kept in the dark ; it must at no time be exposed to direct sunlight.

The amount of methylene blue required for a day's work must be poured off from the stock bottle into a suitable glass container. The pipette used for transferring the methylene blue solution to the tubes of milk must not be introduced into the stock bottle.

The Phosphatase Test

Reagents

Buffer-substrate. Buffer-substrate solution must be prepared at the strength of 1·09 gm. of disodium phenyl phosphate and 11·54 gm. of sodium diethyl barbiturate in 1 litre of distilled water saturated with chloroform. Alternatively, buffer-substrate tablets may be used to make up a solution of the same strength and a few drops of chloroform added. The solutions must be kept in a cool, dark place, and must not be kept longer than three days.

Test reagent. Add 1 volume of Folin and Ciocalteu's reagent to 2 volumes of a 5 per cent. solution of sodium *hexametaphosphate.*

Method of carrying out the test

To 10 ml. of the buffer-substrate solution contained in a test-tube, add 0·5 ml. of well-mixed milk. Add 3 drops of chloroform, stopper the tube, mix the contents and incubate at 37 ± 1° C. for 24 ± 2 hours. At the end of this time, cool, add 4·5 ml. of the test reagent, mix, allow to stand for three to five minutes, and filter into a test-tube marked at 10 ml. To 10 ml. of the filtrate, add 2 ml. of a 14 per cent. solution of pure anhydrous sodium carbonate, mix and place the test-tube for exactly two minutes in boiling water (kept boiling). Cool and read the colour, using a comparator or a tintometer.

Control Tests Keep the remainder of all milk samples in the refrigerator. *After completing the test* carry out control tests on those samples which have given a positive phosphatase reaction.

Mix thoroughly 10 ml. of the buffer-substrate solution with 4·5 ml. of the test reagent, add 0·5 ml. of milk and mix. Allow to stand for three to five minutes, and filter into a test-tube marked at 10 ml. To 10 ml. of the filtrate, add 2 ml. of the sodium carbonate solution, mix and place the tube for exactly two minutes in a boiling water-bath (kept boiling). Cool and read the colour developed. The colour must not exceed 1·5 Lovibond blue units.

Precautions

(*a*) Phenols, disinfectants containing phenols, and soap containing carbolic acid must be kept at a safe distance from the test reagents and apparatus.

(*b*) the use of bottle caps made from phenolic resins must be avoided.

(*c*) new rubber stoppers must be tested for phenolic impurities before use ;

(*d*) all glassware must be clean ;

(*e*) contamination of pipettes by saliva must be avoided ;

(*f*) a fresh pipette must be used for each sample of milk ;

(*g*) all reagents must be kept in a cool, dark place and well protected from dust ;

(*h*) tests must not be carried out in direct sunlight ;

(*i*) freshly boiled distilled water must be used throughout ;

(*j*) samples which show a taint or clot on boiling must not be tested.

Test of reagents

The purity of the reagents must be tested by performing a blank test without milk, with each batch of samples tested. The colour must not exceed 0·5 Lovibond blue units.

For confirmatory tests of coliforms and other tests, see "Water Analysis", page 198.

Estimating the Approximate Number of Viable Bacteria Present by Counting Colonies under the Microscope

The table given below is applicable under the following standard conditions :

(*a*) Internal diameter of dish—9 cm.

(*b*) 0·1 ml. of milk suspended in 10 ml. of medium incubated for forty-eight hours at 37° C.

(*c*) Using the 16 mm. objective, ×4 ocular and a "field" diameter of 0·022 cm. and standard tube-length of 160 mm.

Average number of colonies per field (counting approx. 20 fields)	Approximate number of organisms per millilitre of milk
1	16,200
2	32,400
3	48,600
4	64,800
5	81,000
6	97,200
7	113,400
8	129,600
9	145,800
10	162,000

Examination of Milk for M. tuberculosis

Method A. 100 ml. of the sample (well mixed) is distributed into 50 ml. or 25 ml. centrifuge tubes and centrifuged at 3,000 r.p.m. for thirty minutes. The layer of cream which forms on the surface of each tube is lifted off and placed in a small sterile beaker. The fluid is carefully decanted off and the deposit from all the tubes suspended in 2 or 3 ml. of N/saline solution. Films are made from this suspension and the remainder kept for inoculation into guinea-pigs In this method the films should be washed with ether prior to staining to remove the traces of fat. The cream may be shaken up with a few mls. of ether and re-centrifuged, films being made from the deposit.

Method B 100 ml of sample (well mixed) is heated to 45° C. for fifteen minutes, cooled and rendered faintly alkaline to litmus. 1 ml of liquor trypsini co. is added and the milk incubated at 37° C for two hours, shaking occasionally. The milk is distributed into two 50-ml. or four 25-ml centrifuge tubes and centrifuged for thirty minutes at 3,000 r.p.m. The solid disc of fat which forms on the surface of each tube is lifted off with a sterile spatula, the fluid carefully decanted off and the deposit in each tube [illegible] This is [illegible] a [illegible] of [illegible] guinea-pigs. [illegible] ml. of N/saline for inoculation into

CHAPTER XXIV

BACTERIOLOGICAL EXAMINATION OF SHELL-FISH

VARIOUS types of shell-fish may be received at the laboratory for bacteriological examination, but as the technique is very similar in all instances, that for mussels is given here. This technique is readily adaptable for most other shell-fish.

Apparatus required:

(*a*) A large enamelled bowl.
(*b*) At least two wooden-handled spatulas (ordinary table-knives serve the purpose).
(*c*) Water-bath containing boiling water.
(*d*) Sterile petri-dishes.
(*e*) Sterile test-tubes.
(*f*) Sterile 1-ml. graduated pipettes.
(*g*) Squares of lint (approximately 6 in.).
(*h*) Rectified methylated spirit.
(*i*) Tubes of litmus-milk medium.
(*j*) Lactose-bile-salt agar plates.
(*k*) Agar stabs (10 ml.).
(*l*) MacConkey's fluid medium tubes (single strength).
(*m*) Petroleum jelly.

Ten average-sized mussels selected from the sample are placed in the enamelled bowl under running water for fifteen minutes. The fish are then removed and a square of lint, soaked in methylated spirit is wrapped round each fish. Each fish is unwrapped, opened by inserting a sterile spatula between the shells and twisting sharply, and the contents scraped out into a sterile petri-dish (a separate dish is used for each fish). The shells are discarded. From each petri-dish 0·2 ml. of the liquor is measured into a tube of MacConkey's fluid medium, 0·2 ml. into a test-tube labelled "POOLED LIQUOR", and 1 ml. into a litmus-milk tube. From the "POOLED LIQUOR" tube, now containing a representative fraction from all ten fish,

0·25 ml. is pipetted on to the surface of a lactose-bile-salt agar plate and spread over the surface. The remainder of the "Pooled liquor" is diluted 1:10 and 1 ml. plated out in agar in each of two plates as for counting the viable bacteria in water samples (page 195). Colonies forming in these plates are counted after incubation at 37° C and room-temperature, and the number of organisms per mussel calculated. The litmus-milk tubes are heated at 80° C. for fifteen minutes, hot petroleum jelly run on to the surface of the medium in each tube, and incubated at 37° C. for forty-eight hours; after which they are examined for the presence of clostridium welchii (stormy clot reaction, page 202) The tubes of MacConkey's fluid medium are incubated at 37° C. for twenty-four hours and examined as for E. coli in water samples (page 198).

CHAPTER XXV

A TECHNIQUE FOR THE ROUTINE BACTERIOLOGICAL EXAMINATION OF STOOLS

A STANDARDIZED method for the collection of samples for despatch to the laboratory should be encouraged and suitable receptacles supplied by the laboratory for this purpose (see page 31). Further, it should be made quite clear, preferably by small printed labels on the receptacles, that they should not be more than one-third filled.

Apparatus required:

(*a*) Tubes containing 10 ml. sterile N/saline solution.
(*b*) Tubes containing 5 ml. sterile peptone water.
(*c*) 1:10,000 brilliant green solution (brilliant green sulphate, zinc-free, should be used).
(*d*) Freshly poured plates of the media chosen (see "Culture Media"). (It is an advantage to use several specific media in parallel.)
(*e*) 5-in. glass pipettes with short, wide nozzles.
(*f*) Rubber teats.
(*g*) Micro-slides.
(*h*) A Dreyer dropping pipette.

Tubes of tetrathionate broth and/or selenite F enrichment medium may be included if desired (see "Culture Media", pages 170 and 171).

Technique. *First day.* A 10-ml. tube of sterile N/saline is poured into each specimen and the stool emulsified with the aid of the spoon attached to the cork. (Smaller quantities of saline are used in proportion to the consistency and quantity of the specimen.) With a wide-nozzle pipette approximately 0·5 ml. of the emulsion is transferred to each of two tubes of peptone water and to tubes of any special enrichment media selected, and mixed well. With the same pipette a very small

quantity of the peptone water emulsion is taken and a droplet placed on (*a*) the margin of a plate of each of the specific media chosen and (*b*) the centre of each of two micro-slides. The pipette is then dropped into the lysol bucket. With a glass-spreader (see page 109) the droplet on each plate is spread over the surface of the medium.

Even dilution, giving discreet colonies can be obtained by adopting one of the methods described on pages 112 and 113.

With the Dreyer pipette five drops of the 1:10,000 brilliant green solution are added to one of the peptone water tubes. The peptone water tubes and any others used, are then labelled and placed in the incubator at 37° C., together with the plate cultures. A loopful of weak methylene blue solution is mixed with the emulsion on one of the micro-slides and a loopful of Lugol's iodine solution mixed with the other ; cover-slips are placed over the mixtures and the edges of the cover-slips sealed with petroleum jelly (or other sealing medium to choice). The slides are examined under the 16-mm. objective and then under the 4-mm. objective for the presence of inflammatory cells and other abnormalities. (For " Amoebae ", see pages 258-261.)

Note. In the writer's view it is important to include the plain peptone water tube against the possibility of errors occurring in the preparation of enrichment media which may do more than just inhibit coliform organisms.

Second day. One plate of each of the media chosen is divided into halves by marking with a grease-pencil. One half of each plate is labelled " PEPTONE WATER " and the other " BRILLIANT GREEN ", extra plates will be required for plating out additional enrichment tubes. The inoculated tubes are shaken by gently rotating them between the palms of the hands and a loopful of each spread over the surface of the suitably-labelled halves of the plates. These plates are placed in the 37° C. incubator. The primary plate cultures are searched with a hand-lens for suspicious colonies of which representative colonies are picked off with a platinum needle and transferred to " lactose " sugar tubes (see " Culture Media ", page 149), which are then placed in the incubator

Same evening. The " lactose " tubes are examined, discarding any which show fermentation. The remainder are

sub-cultured into sets of sugar media (glucose, manitol, dulcitol and sucrose) and on to an agar slope. These are placed in the 37° C. incubator.

Third day. The secondary plates are searched and the same procedure as on the second day adopted with any suspicious colonies. The sets of "sugars" are examined and, where the organisms show the reactions of known pathogenic bacteria, the growth on the corresponding agar slope is emulsified in N/saline solution and titrated against the appropriate standard agglutinating sera. The remainder of the sets of "sugars" except those which now show fermentation of lactose, are returned to the incubator.

Fourth day. The sets of "sugars" from the secondary plates together with any remaining from the primary plates are dealt with as before. It is wise to make a final inspection of all plates that have been standing aside from the first day to make sure that no possible pathogenic colonies have been overlooked before discarding.

The advantage of using a number of specific media in parallel is that a check is maintained upon the preparation of each, interesting and instructive comparisons between various media recommended by different workers can be made and the technician becomes familiar with characteristics displayed by organisms growing on the various media. He is therefore able to adapt his technique at short notice to the requirements of others should necessity arise.

CHAPTER XXVI

A TECHNIQUE FOR THE ROUTINE EXAMINATION OF LARGE NUMBERS OF THROAT SWABS FOR C. DIPHTHERIAE AND OTHER ORGANISMS

WHILST seldom specifically asked for by medical practitioners, the presence of other pathogenic organisms in throat swabs submitted to the laboratory for examination for presence of C. diphtheriae, if reported, can be of great assistance to them in subsequent diagnosis.

To include a means of identifying such organisms in a routine technique obviously has its advantages and earns for the laboratory the respect and even perhaps the gratitude of the practitioners concerned if notification of their presence is included in reports.

Apparatus required:

(*a*) Test-tube racks (set out as shown in Fig 46).
(*b*) Blood-agar plates (freshly poured).
(*c*) Löffler's serum slopes.

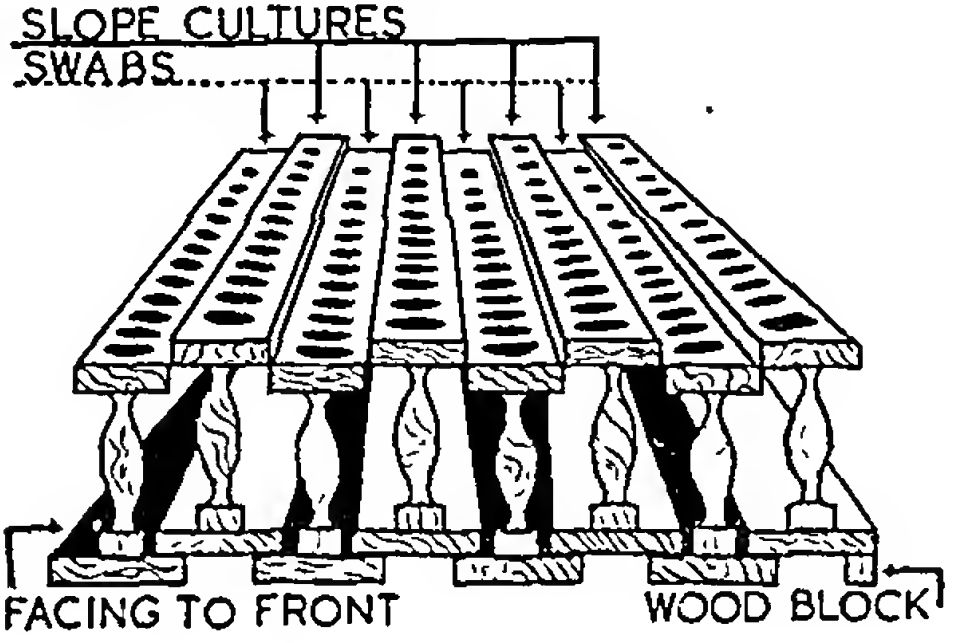


FIG 46.

(*d*) Specific media (to choice).
(*e*) Normal saline solution.
(*f*) Sterile capillary pipettes (with teats).
(*g*) Platinum loop and wire.
(*h*) Strong rubber bands (approximately 1 in. diameter).
(*i*) Micro-slides, grease-pencil and coloured crayon.

The various stains will, of course, be available at the staining sink.

It is, doubtlessly, universal practice for all specimens to receive a serial number prior to arrival at the bench. As time goes on even if numbering is repeated annually, serial numbers become somewhat unwieldy as the total number of specimens received mounts up. These numbers soon become a little "overpowering" for labelling plates and slope cultures, particularly when, as in this technique, a number of implants are made on one plate.

Each day, therefore, the swabs received are numbered consecutively from No. 1 onwards with coloured crayon (preferably RED). The date of receipt and the serial number will subsequently prevent any confusion between batches of swabs.

The blood agar and specific medium plates are each divided into eight segments (either by marking with grease pencil or with the agar-gouge described on pages 109 and 110) and the date plainly written in the centre of each plate.

The segments are numbered consecutively from No. 1 onwards. With a little practice numbers can be written backwards so that they appear, right way round, through the medium when the plates are laid on the bench, face upwards, for inoculation and subsequent searching (see Fig. 22b, page 114).

Commencing with No. 1, each swab, in turn, is washed out thoroughly in the "water of condensation" of a Löffler slope (if none is present a drop of N/saline is added with a sterile pipette). Using the swab, this emulsion is spread over the surface of the slope. It is safe to assume that any part of the emulsion will now contain a representative fraction of all the organisms present (particularly is this so with regard to C. diphtheriae in view of the great ease with which this organism emulsifies). Using the swab, a small drop of the emulsion is placed at the narrow end of its correspondingly

numbered segment of each of two plates (blood agar and specific medium). The plug of the Loffler slope is replaced, as also is the swab in its tube. A rubber band is placed round both swab and slope which, with the remainder of the swabs and slopes, after similar treatment, are stacked in baskets in the 37° C. incubator. Each plate will eventually have eight separate implants, or part thereof, on it. With each implant in turn, the droplet is touched with a glass spreader and drawn out towards the wide end of its segment ; then, starting from the wide end the spreader is drawn backwards and forwards across the segment gradually converging upon the site of the original implant. This " dilution " will almost invariably result in discreet colonies being formed at the wide end of the segment. When all plates have been dealt with, they are placed in the 37° C. incubator.

The following day. The rubber bands are removed from the slopes and swabs, one at a time, and the swabs placed out, in numerical order in the *lower rack* of each pair of racks, with the corresponding slopes in the *raised rack* immediately behind the swabs (see arrangement of racks, Fig. 46, page 217). With a grease-pencil a series of micro-slides are each divided into twelve divisions, labelling so that numbers read clockwise when in use (as in Fig. 22a, page 114), two slides being used for each set of twelve or part thereof.

In turn, never removing more than one slope at a time, the growth on the surface of each slope is emulsified in the " water of condensation " (or a little saline added with a sterile pipette) with a platinum loop and a loopful transferred to the correspondingly numbered divisions on the two slides, where it is spread into thin films. As divisions on the slides correspond with the number of tubes in each rack, any error will become immediately obvious as the end of each rack is reached. When all slopes have been dealt with the slides are dried and heat-fixed in the usual manner. One set of films is stained by a specific stain and the other by Gram's method.

The blood-agar plates are examined for the possible presence of haemolytic streptococci (and possible C. diphtheriae colonies !) whilst the specific medium also receives primary examination, after which it is returned to the incubator for

a further twenty-four hours and again searched. As isolated colonies will be available on the specific medium (and probably on the blood agar too) subsequent typing or virulence tests, if required, will not be held up whilst the mixed growth from the Löffler slope is sub-cultivated for isolation of the organisms thereon. Colonies may be picked off the plates and sub-cultured on to fresh Löffler slopes.

CHAPTER XXVII

A TECHNIQUE FOR ROUTINE EXAMINATION OF LARGE NUMBERS OF SPUTA FOR THE PRESENCE OF M. TUBERCULOSIS

In many Public Health laboratories and at certain Institutions as many as 200 or more sputa may be received each week for examination for M. tuberculosis. With the recent introduction of Chest Radiography Centres even greater numbers may be anticipated. It is reasonably safe to assume that amongst "positive" sputa received from this latter source a high proportion may contain but scanty numbers of tubercle bacilli, so that reports given upon "direct" film preparations alone will prove of little value.

Under the circumstances, therefore, a technique which will ensure safe, speedy yet reasonably reliable results is to be desired.

The equipment required is available in practically every laboratory; it is upon the systematic method of dealing with the sputa and the standardization of methods employed that the technique depends for its rapidity and success. With other urgent work to be "fitted in", much of which—throat swabs, for example—must of necessity be a daily service, the technique must provide opportunity for the technician to break off at short notice yet be able to return to the task knowing just how far it has progressed. Nothing must spoil if left.

The personal safety of the technician must also be considered! The system must be self-checking, by which is meant that any error which may possibly occur must be immediately self-evident and afford opportunity to trace and rectify it without overmuch loss of time and labour.

Apparatus required

(*a*) A large staining sink (at least 1 ft. 6 in. × 1 ft.).

(*b*) A drying-plate. The copper interior of a discarded incubator or similar vessel, with a small section cut

from one side through which can pass the tubing for a small bunsen burner, and with rows of small holes pierced in the side just below the top, is quite suitable (Fig. 47).

(*c*) Two tall, straight-sided glass jars (old museum jars), one half-filled with 5 per cent. lysol solution and the other containing a small quantity of pure lysol.

(*d*) A large number of sturdy glass pipettes about 5 in.

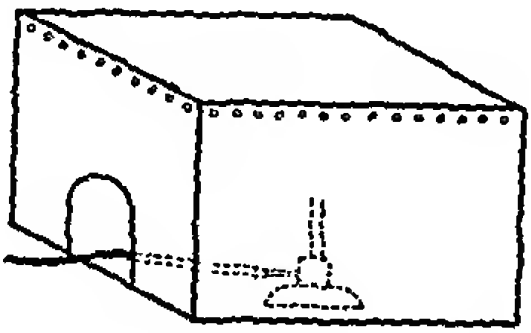


Fig 47. Drying plate for micro-slides

in length and with short, wide nozzles (100 or so should meet average requirements).

(*e*) Rubber teats.

(*f*) Six porcelain staining dishes, with slots to hold six slides in each, filled with 25 per cent. or 20 per cent. sulphuric acid solution.

(*g*) Several shallow wooden trays measuring 12 in. × 6 in. (plain pieces of plywood cut to this size will do).

(*h*) A bottle of about 1 litre capacity labelled:

SODA-PHENOL SOLUTION
(ready for use).

(*i*) Two or three shallow enamelled dishes which will fit into the incubator.

(*j*) Stock bottle containing 10 per cent. sodium carbonate.

(*k*) Phenol (pure).

(*l*) 25 per cent. or 20 per cent. sulphuric acid solution.

(*m*) Hydrochloric acid (pure).

(*n*) Rectified spirit.

(*o*) Under the bench : a large enamelled or galvanized bucket containing 5 per cent. lysol solution.

The stock sodium carbonate solution is diluted with distilled water to 2 per cent and 0·5 per cent. phenol added in the litre bottle daily for use as a concentrating fluid

Technique A. Each evening the sputa received during the day are collected together and, where excess amounts of sputum have been sent, the excess (leaving the bottle about one-third full) is poured into the jar containing pure lysol. All bottles are then filled with the concentrating fluid (soda-phenol), the corks replaced loosely to allow for expansion in the incubator, the bottles packed into the shallow enamelled trays and placed in the incubator at 37° C overnight.

Next morning the trays are removed and placed (to take their turn in numerical order of the laboratory serial number of the sputa) in rows of 18, one row behind the other. The FIRST BOTTLE *in each row is marked in a prominent colour* with the *row number*, using numbers 1 to 10 only (it is safe to assume that after number 10 has been reached the next row can safely revert to number 1 without fear of confusion between rows

We then have a series of rows of bottles, each row numbered from front to back, and each bottle bearing its laboratory serial number. Any transposition of bottles between rows or within their own row would be immediately evident.

Eighteen slides are marked with grease-pencil or a writing diamond Nos. 1 to 18, and placed out in this order on the drying plate. Commencing with the first bottle in the front row, the cork is removed and the supernatant carefully poured off into the jar containing the pure lysol. With one of the pipettes, fitted with a rubber teat, the deposit is stirred thoroughly by drawing it up and expelling it several times ; a small quantity is expelled on one end of the corresponding slide and, using the side of the pipette as a spreader, a thin film is spread over the surface of the slide, leaving a clear margin at the numbered end for handling the slide subsequently. The used pipette is dropped into a jar of 20 per cent. lysol solution.

When all films have been made from the row of eighteen bottles and are thoroughly dried, each in turn is passed rapidly through the bunsen flame to fix it and placed on the staining racks, maintaining the same numerical order. With a gentle

flow of water from a short length of rubber tubing attached to the tap the slides are flooded to remove traces of soda. The water is drained off and the films stained by Zeihl Neelsen's method (see page 238).

The fuchsin is followed by a washing with acid alcohol (3 per cent. hydrochloric acid in rectified spirit) and the slides washed in running water. The slides are then transferred, one at a time and still maintaining their numerical order, to the porcelain dishes containing 20 per cent. or 25 per cent. sulphuric acid according to choice, where they are left for at least twenty minutes (or longer if traces of pink persist after washing). The slides are returned to the staining racks, washed thoroughly with running water and counter-stained with whatever counter-stain is chosen (methylene blue is not to be recommended owing to the risk of masking acid-fast bacilli under blue-stained streaks of debris). Again the slides are washed in running water, drained and transferred, still in the same order, and moving one slide at a time only, to a sheet of blotting paper, prominently marked with the ROW NUMBER, and laid on one of the shallow wooden trays. The films must not be blotted, but placed in a warm atmosphere (the incubator) to dry.

Any damage to the grease-pencil numbers on the slides is repaired before commencing to examine the slides. "Negative" films are discarded as they are finished with; "Positive" films are marked with grease-pencil, a double cross for "numerous" and a single cross for "scanty" and left on the tray until results have been recorded.

Technique B (modification of Petroff's method). The sputa are placed out in rows of 18 as for Technique A, but without any previous concentration and direct films made and stained by Zeihl Neelsen's method. Definite "*positive*" sputa, after recording results, are discarded and the "negative" sputa set aside for further investigation. Of these apparently negative sputa all excess of sputum is removed, leaving the bottles no more than quarter full. The bottles are then filled with 4 per cent. sodium hydroxide solution and placed in the 37° C. incubator for half an hour, shaking occasionally during this period. The 18-per-row system is adhered to for laying out these bottles. Sterile conical centrifuge tubes are

placed out in a series of test-tube racks and labelled with the serial numbers of the sputa. These are filled with the mixtures from their corresponding bottles, centrifuged at 3,000 r.p.m. for thirty minutes and the supernatant fluids carefully poured off into the jar containing pure lysol. The deposits are neutralized with 8 per cent. hydrochloric acid, added drop by drop and controlling the reaction by adding one drop of phenol red indicator to each tube. The neutralized deposits are then inoculated on to Petroff's medium (or other medium according to choice) and films made and stained by Ziehl Neelsen's method in the usual way.

Notes on the above techniques :

(1) The jar used for surplus sputum and supernatants from treated sputa, together with the used pipettes, should be replaced daily with a fresh jar and pipettes The used pipettes, after decanting off the bulk of the lysol solution are autoclaved and washed thoroughly under running water. They are then boiled for one hour in either (*a*) chromic acid

with others, but must be burned.

(3) It is very rare indeed to encounter a sputum which does not break down completely under one or other of the above treatments; should this happen the bottle is set aside as rows are being completed (maintaining a strict eighteen bottles per row) To the bottles that have been set aside a small quantity of Liq. trypsin Co is added and the bottles returned to the incubator, after which they may be included in subsequent batches of sputa

(4) The " self-checking " referred to earlier in the text becomes obvious at each stage of the process For example, when the last bottle in any row is being dealt with, *No. 18 slide* must have been reached and all other specimens in that row must, in consequence, have been smeared on their correct slides. The porcelain dishes must have all compartments filled by the time that the staining rack is emptied Transposition of slides is impossible as they are moved one at a time only in numerical order.

(5) In Method A, when adding the soda-phenol solution it is unnecessary to shake the bottles (a dangerous practice anyway! in view of possible leaky corks) They can be gently rotated to make sure that the sputum is floating freely on the surface of the concentrating fluid.

(6) In Method A great care should be taken to avoid disturbing the deposits when removing the bottles from the incubator to the bench and when pouring off the supernatant fluids.

Preliminary experiments with sulphonated fatty alcohols and with a commercial product (D10, Messrs. Boots Ltd.) lead the writer to believe that such substances may hold advantages over the older methods for concentration of sputa and similar specimens. Sufficient work has not been accomplished, however, to justify more than this passing reference.

SPECIAL NOTE.—**It is unwise to risk the use of old slides for this work as it is almost impossible, in mass-cleaning of slides, to be sure that all M. tuberculosis are removed from "positive" slides. New slides should be used.**

CHAPTER XXVIII

EXAMINATION OF HAIRS AND SKIN-SCRAPINGS FOR RINGWORM

Method A. Make a thin ring of petroleum jelly on a slide and place the fragments of hair or skin-scraping within it. Add a drop of 10 per cent. caustic soda solution and cover with a cover-slip so that the petroleum jelly seals the soda between the slide and cover-slip. Allow to stand for fifteen to thirty minutes before examining.

Note.—Where specimens have been allowed to get dry the strength of the caustic soda solution may be increased to 20–30 per cent.

Method B (Tribondeau's method). Place a number of hair fragments or skin scrapings in a small centrifuge tube and cover with ether. Centrifuge, pour off the ether and add an equal volume of alcohol. Centrifuge, replace the alcohol with water and again centrifuge. Suspend the deposit in a drop of 10 per cent. caustic soda solution on a slide, cover with a cover-slip and allow to stand for one hour. Run glycerine under the cover-slip by placing a drop touching one side of the mount and holding a piece of filter paper at the opposite side so drawing the glycerine under the cover-slip as the soda is absorbed by the paper.

Method C (Stitt, Clough and Clough). Fragments of hair or skin-scrapings are immersed in liquid paraffin on a slide and covered with a cover-slip. After a while hyphae of the fungus grow out of the fragments into the paraffin.

Method D. Treat fragments of hair with ether in a watch-glass. With fine-pointed forceps transfer the hairs to a fresh watch-glass containing 10 per cent. caustic potash solution and allow to stand for ten to fifteen minutes. Again transfer the hairs to a watch-glass containing distilled water and allow to stand for a further ten to fifteen minutes. Place a small drop of Lacto-phenol solution (with cotton-blue) on a slide,

immerse the fragments of hair in this and cover with a cover-slip. The structure of the hair and the hyphae of the fungus will be stained varying shades of blue. The spores will show as clear transparent bodies.

By this method permanent preparations may be produced and the cover-slips sealed with a suitable " ringing " medium.

CHAPTER XXIX

SLIDE PREPARATIONS FOR MICROSCOPICAL EXAMINATION

(WET PREPARATIONS, STAINS AND STAINING METHODS, ETC.)

In order to study bacteria in the living state it is necessary to employ either " hanging drop " or plain wet preparations, but much can be learned from films made from cultures and other material and stained by special methods, in fact, in certain instances the reaction of bacteria to dyes and combinations of dyes may be diagnostic.

Wet Preparations

Hanging drop preparations

Apparatus required :

Welled slides
Cover-slips
Petroleum jelly
Sterile N/saline

The glassware must be perfectly clean and free from grease. With a sterilized platinum loop a small drop of young broth culture or thin emulsion in N/saline of the bacteria from a colony on solid medium, is placed on the centre of a cover-slip. The margin of the depression on a welled slide is ringed with petroleum jelly and the slide inverted over the cover-slip so that the drop of culture is in the centre of the depression. The slide is gently pressed down, causing the petroleum jelly to adhere to the cover-slip, after which it is turned over when the drop of culture will be suspended in the well of the slide.

" Hanging drop " preparations should always be employed for demonstrating motility of organisms; in addition to the risk of possible convection currents in the fluid giving an impression of motility, oxygen starvation may slow down or completely inhibit true motility in ordinary wet preparations.

Wet films. Using a plain slide in place of the welled variety, the above technique is repeated, making a thin ring of petroleum jelly on the slide slightly smaller than the cover-slip. The slide is pressed on to the cover-slip until the drop of culture spreads out into an even film between the cover-slip and slide, but is sealed off by the ring of petroleum jelly.

Intra-vital staining. To stain bacteria in wet preparations, a minute quantity of dilute methylene blue solution, brilliant cresyl blue or other chosen stain may be introduced into the drop of culture prior to sealing on to the slide. Alternatively, a clean grease-free slide may be flooded with a thin film of methylene blue solution or 0·5 per cent. alcoholic brilliant cresyl blue and allowed to dry. The stained side of the slide is used upon which to mount the wet preparation. The minute amount of stain with which the drop of culture comes into contact will be just sufficient to stain the bacteria as it redissolves in the fluid.

Film Preparations

To prepare a film of bacterial growth or material containing bacteria, a loopful of either fluid culture, suspension of material in N/saline or thin emulsion of bacterial growth from solid medium in N/saline is taken and spread evenly over the surface of a slide or cover-slip. The film is allowed to dry in air, or in a warm current of air by holding it high over a bunsen flame, and is then fixed by passing the slide or cover-slip rapidly through the bunsen flame three times, film-side uppermost. Heat-fixation is limited in its application, and, where cellular structure is to be studied, other methods of fixation must be employed.

Methods for Demonstrating Fat in Micro-organisms

Sudan III

Sudan III	0·1 gram.
50:50 glycerol and alcohol	1,000 ml.

Wet preparations of the organisms are made with the solution as the mountant. Fat globules are stained red.

Osmic acid

Osmic acid · · · ·	0·1 gram.
Distilled water · · ·	100 ml.

(The solution must be kept in a well-stoppered dark bottle.) Wet preparations of the organisms are made in the solution. Fat globules are stained dark brown to black.

NOTE—Great care must be exercised when using the solution as dangerous fumes are given off when it is exposed to air.

Flemming's solution

1 per cent. osmic acid solution	15 parts.
Acetic acid (glacial) · ·	1 part.
2 per cent. chromic acid solution	4 parts.

Wet preparations are made with the solution as the mountant. Fat globules are stained black.

A Method for Demonstrating Volutin in Micro-organisms

An emulsion of the organism in formol saline is allowed to stand for several hours after which it is centrifuged, films prepared from the deposit and dried in air.

A drop of:

Methylene blue · · ·	1 gram.
25 per cent. alcohol · ·	100 ml.

is placed on the film and covered with a cover-glass. 1 per cent. sulphuric acid solution is placed at one edge of the cover-glass and drawn under it by touching the opposite edge with a strip of blotting paper. Volutin granules appear as bright blue bodies in the decolorized organisms.

Mounting Media for Slide Preparations

Canada balsam

This is a natural resin and requires to be dissolved in pure xylol to the consistency of thick cedar-wood oil for use The disadvantage is that it is acid in reaction and tends to bleach stained preparations.

Euparol

Similar in appearance and general use to Canada balsam. The suppliers (Messrs. Flatters & Garnett Ltd., Manchester) claim that it does not affect stained preparations.

"D.P.X."

Dibutyl phthalate . .	5 ml.
Pure xylol	35 „
"Distrene 80" . . .	10 grams.

("Distrene 80" can be obtained from Messrs. Honeywell & Stein Ltd., 21 St. James's Square, London, S.W.1.)

CHAPTER XXX

STAINS, STAINING METHODS AND FIXATIVES

Simple Stains

Methylene blue solution

Methylene blue	1 gram.
Distilled water	100 ml.

Dissolve the powder in the water and filter before use.

Methylene blue (Loffler's)

Saturated alcoholic solution of methylene blue	30 ml.
1:10,000 potassium hydroxide solution	100 „

(two drops, approximately 0·1 ml., of 10 per cent. KOH in 100 ml. water makes a 1:10,000 dilution).

Polychrome methylene blue

Loffler's methylene blue is allowed to " ripen " by standing over a period of several months, shaking at intervals to aerate the solution, until it is of a violet colour, or air may be drawn slowly through the solution by means of a vacuum pump.

Carbol thionin

50 per cent. alcoholic solution of thionin	10 ml.
2 per cent. phenol solution	100 „

Methyl green-pyronin (Pappenheim, modified)

Methyl green	0·15 gram.
Pyronin	0·5 „
95 per cent. alcohol	5 ml.
Glycerin	20 „
2 per cent. phenol solution	75 „

Archibald's stain

Solution No. 1 :

Thionin	0·01 gram.
Formalin	1 ml.
2·5 per cent. phenol solution	100 „

Solution No. 2 :

Methylene blue	0·5 gram.
Formalin	1 ml.
2·5 per cent. phenol solution	100 „

The two solutions are kept separately and must stand for at least twenty-four hours before use. For use, equal parts of each are mixed and filtered.

Dilute carbol fuchsin (A)

Carbol fuchsin (as for Ziehl Neelsen's stain)	10 ml.
Distilled water	100 „

Dilute fuchsin (B)

Basic fuchsin	0·5 gram.
Distilled water	1,000 ml.

Staining Methods

Gram's stain

Many modifications of Gram's staining method have been introduced and choices vary considerably. The principle is the same in all instances, that is, to divide all stainable bacteria into two distinct groups : " Gram Positive " and " Gram Negative ".

Gram positive organisms retain the purple dye and appear purple or purple-black in films, whilst Gram negative organisms are decolorized during the process of the technique and take up the colour used as a counterstain.

Weigert's modification

1. (*a*) Gentian violet (or crystal violet)	2 grams.
Aniline	9 ml.
95 per cent. alcohol	33 „
(*b*) Gentian violet (or crystal violet)	2 grams.
Distilled water	100 ml.

For use, mix one volume of (*a*) with nine volumes of (*b*) and filter. The mixture will keep for two to three weeks only.

Note.—Various workers prefer :

Saturated alcoholic gentian violet solution	1 vol.
5 per cent. phenol solution	10 vols.

(mix and filter for use).

or

Methyl violet (6B)	0·5 grams.
Distilled water	100 ml.

or

Saturated alcoholic solution of crystal violet	25 ml.
1 per cent. sodium oxalate solution	100 ,,

(this stains deeply and requires 30 seconds only).

2. Grams Iodine Solution

Iodine (resublimed)	1 gram.
Potassium iodide	2 grams.
Distilled water	300 ml.

(the potassium iodide is dissolved in 5 ml. of the water and the iodine dissolved in this concentrated solution before adding the remainder of the water).

3. Aniline-xylol

Aniline	2 vols.
Xylol	1 vol.

4. Counterstain

Carbol Fuchsin (Ziehl Neelsen)	1 vol.
Distilled water	9 vols

Technique

(*a*) Flood the film with the violet solution and allow to remain for three minutes.

(*b*) Wash in water.

(*c*) Flood with iodine solution and allow to remain for one minute.

(*d*) Dry by blotting and then in the air.

(*e*) Decolorize with aniline-xylol using repeated washings until no further colour is removed from the film.

(*f*) Wash with xylol and dry in the air.

(*g*) Counterstain with dilute carbol fuchsin solution for not more than twenty seconds.

(*h*) Wash, blot and dry.

Jensen's modification

1. Methyl violet (6B) . . 0·5 gram.
   Distilled water . . . 100 ml.

(mix and filter. This solution keeps indefinitely.)

2. Lugol's Iodine Solution
   Iodine (resublimed) . . 1 gram.
   Potassium iodide . . 2 grams.
   Distilled water . . . 100 ml.

(make up as for Gram's iodine).

3. Counterstain
   Neutral red . . . 1 gram.
   1 per cent. acetic acid solution 2 ml.
   Distilled water . . . 1,000 „

Technique

(*a*) Flood film with the violet solution and leave for thirty seconds.

(*b*) Tilt the slide and wash off excess of violet with Lugol's iodine solution, then flood with fresh iodine solution and leave for one minute.

(*c*) Wash with rectified spirit until the violet colour ceases to be removed from the film.

(*d*) Wash in water.

(*e*) Counterstain with neutral red solution for one minute.

(*f*) Wash, blot and dry.

Note.—For examining films for the gonococcus, Sandiford recommends the following counterstain :

Malachite green . . . 0·05 gram.
Pyronin . . . . . 0·15 „
Distilled water . . . . 100 ml.

The solution is applied for two minutes and then flooded off with water, avoiding lengthy washing. Cells and nuclei appear bluish-green, Gram positive organisms purplish-black and gonococci bright red.

Kopeloff and Beerman's modification

1.	1 per cent. aqueous methyl violet solution	30 vols.
	5 per cent. sodium bicarbonate solution	8 „
2.	Iodine (resublimed)	1 gram.
	Potassium iodide	2 grams.
	Distilled water	240 ml.

(dissolve as for Gram's iodine and then add 60 ml. of 5 per cent. sodium bicarbonate solution (Kilduffe)).

3. Acetone (as decolorizing agent).
4. Counterstains, a choice of

Saturated alcoholic solution, Safranin (water-soluble)	10 ml.
Distilled water	90 „

or

Dilute carbol fuchsin (as for Weigert's method).

or

Bismark brown	0·2 grams.
Distilled water	100 ml.

(dissolve the Bismark brown in the water by boiling and filter)

or

Basic fuchsin	0·05 grams.
Distilled water	100 ml.

Technique

(*a*) Flood the film with the violet solution and allow to remain for five minutes.

(*b*) Wash with water.

(*c*) Flood the film with iodine solution and leave for two minutes.

(*d*) Drain off the iodine solution and decolorize with acetone (decolorization is very rapid and must be carefully controlled).

(*e*) Wash in water.

(*f*) Counterstain for thirty seconds to one minute.

(*g*) Wash, blot and dry.

Methods for Staining Acid-fast Organisms

Ziehl Neelsen's method (modified) for M. tuberculosis

Solution 1

Carbol fuchsin

Basic fuchsin	1 gram.
Alcohol	10 ml.
5 per cent. phenol solution	100 ml.

The fuchsin is dissolved in the alcohol, using gentle heat (immersion in a water-bath) and the phenol solution added. The solution is filtered before use.

Solution 2

Acid alcohol

Alcohol (rectified spirit)	100 ml.
Hydrochloric acid (pure)	3 ml.

Solution 3

Counterstain

0·1 per cent. methylene blue or
0·1 per cent. malachite green.

Technique

Films are heat-fixed in the usual manner and flooded with the carbol fuchsin solution. A swab of cotton-wool, soaked in rectified spirit is ignited and passed under the row of slides (or slide) until steam rises freely (but the solution does not boil). The films are left to stain for ten minutes. Without pouring off the stain, the slides are flooded with acid-alcohol and left for a further two minutes after which they are flooded with a gentle stream of tap water from a length of rubber tubing fitted to the tap. Using forceps, the slides are placed in a Coplin jar or porcelain staining dish containing sufficient 25 per cent. (or 20 per cent.) sulphuric acid to completely cover the films. The time for decolorization will vary according to the thickness of films and the material being investigated ; films should appear colourless or tinted faintly pink when washed in running water. The slides are returned to the staining rack and counterstained for a few moments only (just sufficiently long to tint the films for the purpose of focussing under the microscope).

Slides are finally washed in running water and air-dried by sloping the slides against a suitable ledge to drain (or they may be laid out on a sheet of filter-paper on the drying plate).

For staining M. leprae the above technique may be applied, but reducing the strength of the sulphuric acid to 5 per cent.

Where there is risk of acid-fast saprophytes becoming involved, the application of a second treatment of acid-alcohol is recommended following the acid-bath. This is of particular importance when examining samples of urine for M. tuberculosis on account of the frequency with which M. smegma is present in these samples. It is of particular importance that distilled water only be used for making up all solutions involved in the technique prior to the actual staining of films; the corrosion which accumulates at the mouths of water-taps frequently contains acid-fast organisms (a fact which can be readily demonstrated by preparing slides from such material from any ordinary water-tap).

Pappenheim's modification of Ziehl Neelsen's method

Immediately following the carbol fuchsin, slides are flooded, without previous washing, with a solution of:

Corallin	1 gram.
Alcohol (abs.)	100 ml.
Methylene blue	0·66 gram.
Glycerol	20 ml.

The films are then treated with several washings of the solution followed by washing in water.

It is claimed that M. smegma is decolorized whilst M. tuberculosis retains the red dye.

Fontes' method

The routine Ziehl Neelsen's technique is carried out using the acid-alcohol only for decolorizing, up to washing after the acid-alcohol. The films are then stained by Gram's method, using bismark brown as a counterstain. It is claimed that this method demonstrates Much granules as deeply stained violet dots and partly damaged tubercle organisms as irregular violet rods against a pale brown background.

C. diphtheriae appear as pale green organisms with darker green banding and blue-black polar bodies.

Methods for Demonstrating Capsules

Hiss's method

Solution No. 1

Saturated alcoholic solution of Gentian violet or Fuchsin	5 ml.
Distilled water	95 „

Solution No. 2

Copper sulphate (cryst)	20 grams.
Distilled water	100 ml.

Technique

Films should be fixed by flooding with alcohol for thirty seconds to one minute. (They should not be heat-fixed.) Allow the alcohol to dry, then flood with solution No. 1 and warm gently until steam rises. Wash off the stain with solution No. 2, do not wash in water. Blot and dry.

McEwen's method

Thin films of the organisms are made on cover-slips and fixed by immersion in alcohol for at least one minute.

Solution No. 1

Carbol fuchsin (Ziehl Neelsen).

Solution No. 2

10 per cent. aqueous solution of tannic acid.

Solution No. 3

1 per cent. aqueous solution methylene blue.

Technique

Flood the films with warm carbol fuchsin and leave for one minute. Wash thoroughly in running water and dry. Invert the cover-slip in a watchglass. Mix equal parts of solutions Nos. 2 and 3 in a test-tube and boil. Then, after allowing the mixture to cool for a few moments, run it into the watch-glass so that the cover-slip floats. Allow to cool and then flood with running water until no trace of blue remains. Dry the film and mount in canada balsam.

Organisms appear deep red to black with pale blue capsules.

Richard Muir's method

Solution No. 1

Carbol fuchsin (Ziehl Neelsen).

Solution No. 2

Saturated solution of mercuric chloride	2 vols.
20 per cent. solution of tannic acid	2 „
Saturated solution of potassium alum	5 „

Solution No. 3

Löffler's methylene blue

Technique

Thin films are fixed by heat in the usual manner and flooded with warm carbol fuchsin solution for one minute The films are then washed in water and flooded with the solution No. 2 (Mordant) for thirty seconds. Again they are washed in water and counterstained with Loffler's methylene blue for one minute. They are finally washed and dried, or dehydrated with alcohol, cleared with xylol and mounted in canada balsam.

Organisms appear red with blue capsules.

Fleming's method

Films are fixed by heat in the usual manner and, with the end of a slide, a thin film of 10 per cent. nigrosin solution is spread over half the film and allowed to dry The films may be stained with weak carbol fuchsin prior to adding the nigrosin film, when capsulated organisms appear as pink bodies with clear halos against a dark background.

Wadsworth's method

Films are fixed in pure formalin for three minutes followed by routine Gram staining. Capsules appear as pale halos surrounding the stained bacteria.

Methods for Demonstrating Flagella

In all methods employed it is important that the delicate hair-like flagella remain undamaged during preparation and

fixation of films. Scrupulously clean slides and cover-slips must be used and all stages of the technique carried out with the greatest possible care.

Preparing films

(*a*) A tube of sterile distilled water is inoculated with sufficient of a young culture of organisms to produce a faint turbidity in the upper part of the tube. This is then incubated for two to three hours at 37° C.

(*b*) Growth from a young agar-culture is emulsified in distilled water to form a turbidity just visible to the naked eye.

(*c*) *Fleming's method.* Small circles of cellophane are autoclaved and kept in sterile distilled water. When required, one circle is placed on the surface of an agar plate and allowed to dry off in the incubator after which the organism to be examined is inoculated on to the surface of the cellophane and the plate incubated for not more than twenty-four hours. The cellophane is then gently prized off the agar, using sterile forceps, placed in a sterile petri-dish and sufficient sterile distilled water added to cover the cellophane. This is incubated at 37° C. for two or three hours when the organisms will partially float free of the cellophane. 1 ml. of formalin is added, drop by drop, to fix the bacteria and the plate left undisturbed overnight. The suspension may then be poured into a small screw-cap bottle and kept for use as required. To make films a small quantity of the suspension is diluted with sterile distilled water.

Thin films are made from the suspension and dried in the air.

Staining Films

Löffler's method

Solution No. 1

Hot 25 per cent. tannic acid solution	16 ml.
Sat. ferrous sulphate solution	10 „
Sat. alcoholic basic fuchsin	2 „

Solution No. 2

Sat. alcoholic basic fuchsin	5 ml.
3 per cent. solution of aniline in distilled water	50 „

Technique

Flood the film with solution No 1 and leave for three to five minutes, wash in distilled water and flood with solution No 2. Warm the slide by passing a flame under it two or three times over a period of three minutes. Wash dry and examine.

Organisms appear as deep red bodies with pale pink to red flagellae.

Peppler's method

Solution No. 1

Tannic acid	20 grams.
Distilled water	80 ml.
2·5 per cent. chromic acid	15 ,,

Solution No. 2

Gentian violet	0·5 gram.
Alcohol	10 ml.
Phenol	2·5 gram.
Distilled water	1,000 ml.

Technique

The method of staining is similar to that for Löffler's method, omitting the warming of the second solution

J. Kirkpatrick's method

Fixative

Absolute alcohol	60 ml.
Chloroform	30 ,,
Formalin	10 ,,

Mordant

Ferric chloride (5 per cent solution)	1 vol.
Tannic acid (20 per cent. solution)	3 vols

(this is diluted with an equal quantity of distilled water when required for use)

Silver Solution

(*a*) Stock.

Silver sulphate	5 grams
Distilled water	100 ml.

(incubate in a well-stoppered bottle for twenty-four hours at 37° C.).

(*b*) Solution for use

Stock silver solution	40 ml.
Ethylamine (33 per cent. W/V)	0·6 „

(When mixed a precipitate appears which immediately redissolves. Stock silver solution is added, drop by drop, until the solution is permanently opalescent ; then 10 ml. of distilled water is added.)

Technique

1. Prepare films of the organisms by gently spreading a loopful of the suspension over a slide and, with the loop, drawing the surplus to one side to form a thick spot which will act as a control for the staining process. Allow the film to dry in the air.
2. Flood the slide with the fixative and allow to remain for three minutes.
3. Rinse the slide with rectified spirit and then wash with water.
4. Flood the slide with mordant and allow this to remain for five minutes.
5. Wash thoroughly with water and drain off all excess.
6. Using a perfectly clean funnel and a Whatman filter paper, filter the silver staining solution on to the slide and warm gently until the "control spot" on the slide becomes dark brown in colour (usually fifteen to twenty seconds).
7. Flood off the staining solution with running water (do not pour off !).
8. Dry and mount in canada balsam.

Plimmer and Pain's method

Mordant

Tannic acid	10 grams.
Aluminium chloride	18 „
Zinc chloride	10 „
Rosaniline HCl.	1·5 „
Alcohol (60 per cent.)	40 ml.

(Place all the solid ingredients in a small mortar, grind well with 10 ml. of the alcohol and then add the remainder of the alcohol. The resultant fluid is viscous and of a deep red colour. It keeps well. For use it is diluted with four times its volume of water when a slight precipitate appears.)

Technique

A small drop of the suspension of organisms is placed at one end of a warm slide (not hot), which is tilted to allow the drop to run down it, the thin film left behind the drop drying almost instantaneously. The mordant is filtered on to the film, allowed to remain for one minute and then washed off with distilled water. The film may be stained subsequently with either methylene blue or weak fuchsin solution.

Fleming's modification of Zettnow's method

Mordant

20 per cent. tannic acid solution · 3 vols.
5 per cent. tartar emetic solution · 2 „

(The precipitate which forms is redissolved by boiling a small quantity in a test-tube at the time it is required.)

Silver Solution

A saturated solution of silver sulphate in distilled water, diluted with an equal volume of distilled water with just sufficient ethylamine added to redissolve the precipitate which first appears.

Technique

Flood the slide with the mordant (heated to almost boiling point) and leave for one minute, followed by rapid washing with distilled water. A small quantity of the silver solution is heated to almost boiling point and the film flooded with it. This is allowed to act for one minute, after which the slide is washed rapidly with running water. The slide is then blotted and dried and immersed in a weak solution of gold chloride for thirty minutes to one hour if permanent preparations are required.

Methods for Demonstrating Spores

Löffler's methylene blue

Flood films with Loffler's methylene blue solution and allow to remain for two or three minutes. Wash, blot and dry.

Spores appear as highly refractile bodies against the blue-stained bacteria.

Modification of Ziehl Neelsen's method for T.B.

The technique is similar to that for demonstrating M. tuberculosis with the exception that 1 per cent. sulphuric acid is used in place of the 25 per cent. solution and films are left in this decolorizing solution for a few minutes only. Films may be counterstained with Löffler's methylene blue or weak malachite green solution. Spores appear pink against the blue bodies of the organisms.

Möller's method

Heat-fixed films are treated with chloroform, washed and flooded with 5 per cent. chromic acid which is allowed to remain for one minute. The films are again washed and then stained by the modification of Ziehl Neelsen's method above.

Fleming's method

Heat-fixed films are stained with hot carbol fuchsin for five minutes, washed and immersed in 1 per cent. nigrosin solution for five to ten minutes (or, alternatively, in 5 per cent. sodium sulphite solution for thirty seconds). The films are washed in water and dried. A nigrosin-film (see Fleming's capsule stain, page 243) is then made across the film. Spores appear as bright red bodies in clear unstained bacteria against a homogeneous dark background.

McFadyean's Stain for B. anthracis in blood films

Films are stained for a few seconds only with polychrome methylene blue solution, washed thoroughly and dried. The presence of purplish fragments between the bacilli indicates the presence of disintegrated capsular material and is characteristic of B. anthracis.

The Romanowski Stains

The original Romanowski stain has been superseded by a number of modifications which are not only easier to use, but give better results.

Giemsa's modification

Azur II (eosin) · · · ·	0·3 gram.
Azur II · · · · ·	0·08 „

dissolve both ingredients in 25 ml. pure anhydrous glycerine at 60° C. and add 25 ml. of absolute methyl alcohol at the same temperature. Allow to stand overnight and filter.

Technique

Films (made on cover-slips for preference) are fixed by immersion in absolute methyl alcohol for approximately three minutes and dried in the air. Cover-slip preparations are inverted in a watchglass and diluted Giemsa's stain (one part stain to 10–15 parts distilled water), buffer solution or alkaline solution (page 251) run into the watchglass to float the film as for McEwen's capsule stain (page 242). Allow to stain for fifteen to thirty minutes according to depth of colour desired, then wash in distilled water until the film becomes pink; dry and mount.

The alkaline diluent is used when it is required to demonstrate coarse stippling in red-blood cells in malignant malaria, or spirochaetes.

Wright's modification

Methylene blue (medically pure) ·	1 gram.
0·5 per cent. sodium carbonate solution · · · · ·	100 ml.

Heat in the steamer for one hour, cool, filter and add :

1:1,000 yellow water-soluble eosin	500 ml.

(add this slowly, stirring all the while until a fine granular black precipitate is formed)

The precipitate is collected on a Whatman filter paper and dried in the incubator (or hot-water oven at 60° C.).

0·1 gram of the powder is dissolved in 60 ml. of pure methyl alcohol and the solution stored in the dark.

Technique

As for Giemsa's method or may also be used as for Leishman's method.

Wilson's modification

Silver nitrate	2 grams.
Distilled water	50 ml.

Dissolve the salt in the water and add 5 per cent. sodium hydroxide drop by drop, until all the silver is precipitated in the form of silver oxide. Wash this precipitate (which is quite heavy) with several washings of distilled water, decanting off each time. 200 ml. of 1 per cent. methylene blue in 0·5 per cent. sodium carbonate solution is added to the moist precipitate in a shallow evaporating basin and the mixture boiled for thirty minutes. One third of the contents of the basin is transferred to a 200 ml. cylinder and a similar amount of boiling distilled water added to the mixture remaining in the dish which is allowed to continue boiling for a further thirty minutes. Again pour one third of the contents into the cylinder and boil the remainder of the solution in the dish for thirty minutes more. Add this to the solution in the cylinder and make up the bulk to 200 ml. Pour the solution into a 500-ml. beaker and add 200 ml. of 0·5 per cent. solution of yellow water-soluble eosin. Allow to stand for thirty minutes and filter through Whatman No 42 filter paper. Dry the precipitate in the incubator. For use, 0·2 gram of the dried powder is dissolved in 50 ml. of absolute methyl alcohol. The solution should be kept in the dark in a well-stoppered bottle.

Technique

Using cover-slip preparations, float the cover-slip film downwards on undiluted stain in a watchglass for one minute and then add an equal volume of diluent (see "Diluents", page 251) and allow to stain for five minutes. Wash the film by running distilled water into the watchglass until all trace of the stain is washed away and the film has a pink tint. Dry by blotting, or in the air.

Leishman's modification

It is advisable to obtain the dry powder commercially.

Place 0·15 gram of the powder in a glass mortar, add a small quantity of absolute methyl alcohol and grind these together.

Pour the alcohol into a clean bottle, add a further small quantity of alcohol to the powder and repeat the grinding, again pouring the alcohol into the bottle. Repeat this until 100 ml. of alcohol have been used and all the powder has been dissolved. The stain must not be filtered, and is better if kept for a few weeks before being brought into use.

Technique

Films are not fixed by heat.

The undiluted stain is poured on to films and allowed to remain for one minute. Double the quantity of diluent as stain is added, drop by drop, from a capillary pipette fitted with a rubber teat and the mixture then sucked up and expelled from the pipette several times to ensure even distribution of stain throughout the diluent. The diluted stain is allowed to act for between five and ten minutes, according to depth of colour required, after which it is flooded off with diluent and a further quantity of uncoloured diluent allowed to remain on until the film loses the bluish tint. (It may be necessary to add plain distilled water instead of diluent to achieve this.) Dry by blotting or tilting the film sideways to drain dry.

Diluents

Most distilled water in the laboratory will be found to be slightly acid in reaction. As any slight variation in its reaction when used as a diluent for the above stains will alter the staining considerably, the water must be neutralized before use.

Where large numbers of films are to be stained by Romanowski methods, a large aspirator should be kept for the express purpose of storing neutralized distilled water. By adding a few drops of phenol red solution to the aspirator the reaction of the water may be controlled by occasional addition of drops of 1 per cent. sodium carbonate solution whenever the colour-tint in the aspirator becomes yellowish. The sodium carbonate solution is added until the contents of the aspirator turn faintly pink. It is a good plan to boil a small quantity of distilled water prior to use, to drive off the dissolved carbon dioxide, and allowing it to cool.

Buffer solution. A solution which is just on the acid side of neutral may be used for diluting the stains if they are alkaline.

Mono-basic potassium phosphate	6·63 grams.
Di-basic sodium phosphate (anhyd.)	2·56 „
Distilled water	1,000 ml.

The pH of this solution should be 6·4.

Methods for Demonstrating Treponemata

Fontana's method (Tribondeau's modification)

Solution No. 1 (Ruge's solution)

Acetic acid (glacial)	1 ml.
Formalin	2 „
Distilled water	100 „

Solution No. 2

Tannic acid	5 grams.
Distilled water	100 ml.

Solution No. 3 (Fontana's solution)

Silver nitrate	5 grams.
Distilled water	100 ml.

(Ammonium hydroxide is added drop by drop until a brown precipitate appears and just redissolves, followed by drops of 5 per cent. silver nitrate until the solution becomes slightly cloudy.)

Technique

Thin films of the material to be examined are made and dried in the air ; they are not heat-fixed. Flood the films with several washings of No. 1 solution over a period of one minute, followed by a few drops of alcohol. The slide is drained and warmed over the bunsen flame to dry rapidly. Flood the films with solution No. 2, warm the slide until steam rises and allow to act for thirty seconds. Wash the slide well with distilled water and flood the slide with solution No. 3, again warm until steam rises and leave for thirty seconds. Wash in distilled water and dry in the air.

Specimens mounted in canada balsam fade rapidly, but films will last longer if mounted in pure liquid paraffin and the cover-slip sealed on the slide with a good ringing medium.

Fontana's method (Becker's modification)

Procedure is the same as in Tribondeau's modification up to the washing following the application of the solution No. 2, after which the films are flooded with

Saturated alcoholic solution of basic fuchsin · · · ·	45 ml.
Shunk's mordant (95 per cent. alcohol 16 ml aniline 4 ml)	18 ml.
Distilled water · · · · ·	100 „

(Add the Shunk's solution to the basic fuchsin and then add the distilled water. Filter before use.)

The films are left in this stain for five minutes, after which they are washed thoroughly in distilled water, dried and mounted in canada balsam (or liquid paraffin).

Tilden's method

(1) Potassium phosphate (0·9 per cent.) 12 ml. · · · Sodium phosphate (2·4 per cent.) 88 ml.	90 ml.
Formalin · · · · ·	10 „
(2) Saturated alcoholic solution basic fuchsin (or gentian violet).	

Technique

A small amount of the exudate or tissue scrapings are suspended in solution No. 1 on a slide, allowed to stand for five minutes and then spread out into a thin film and allowed to dry in air. Flood the film with the alcoholic dye solution, wash off immediately with distilled water and again dry in air.

"Negative" staining method

A small amount of exudate is mixed with a drop of indian ink or 10 per cent. nigrosin and spread into a thin film and allowed to dry. Treponemata appear as unstained organisms against a dark background.

Benion's method

A small drop of 2 per cent. aqueous congo red solution is mixed with a small quantity of the exudate, spread into a thin film and dried in air. The slide or cover-slip is held, film downwards, over the mouth of the concentrated hydrochloric acid bottle until the film turns greenish blue. The film is not washed. Treponemata appear as unstained organisms on a blue background.

Levaditi's method for staining treponemata in tissues

Small pieces of formalin-fixed tissues are washed, first in running tap water and then in distilled for thirty minutes. They are transferred to small dark-glass bottles containing 1·5 per cent. silver nitrate solution and incubated at 37° C. for three days. The tissues are washed in distilled water for thirty minutes and placed in dark-glass bottles containing :

Pyrogallic acid	4 grams.
Formalin	5 ml.
Water	100 „

and left for two days at room temperature. After further washing in running water for at least one hour the tissues are dehydrated, cleared and embedded in paraffin wax as for ordinary sections (see page 189).

The sections, when cut and dried have the wax removed with xylol and are mounted in balsam or euparol.

Jahnel's method for staining treponemata in tissues

Small formalin-fixed pieces of tissue are left in slowly-running water for at least twenty-four hours and then immersed in pure pyridin for three days. This is followed by further washing in repeated changes of water over a period of three days. The tissues are placed in 5 per cent. formalin for a minimum of three days. They are then transferred to distilled water in which they remain for forty-eight hours and then immersed in 1 per cent. uranium nitrate in distilled water and incubated at 37° C. for one hour. They are again washed well in distilled water for twenty-four hours, placed in 96 per cent. alcohol for a week and then returned to distilled water where they remain

until they sink. The tissues are transferred to dark-glass bottles containing 1·5 per cent. silver nitrate solution and incubated for one week. They are next washed in distilled water and immersed for two days in a solution of

Pyrogallic acid (4 per cent.)	85	ml.
Acetone	5	„
Pyridine	15	„

They are finally washed in distilled water and treated as for ordinary paraffin sections (see page 189)

Methods for Demonstrating Yeasts and Moulds

Methylene blue (Yeasts)

0·01 per cent. methylene blue solution.

Technique

Make a thin wet preparation of the yeast and place a small drop of methylene blue solution at one edge of the cover-slip, touch the opposite edge of the cover-slip with filter-paper until the blue commences to penetrate into the preparation. Remove the filter-paper and seal the cover-slip with petroleum jelly. Examine the margin of the blue and colourless fluids.

Lacto-phenol (Moulds)

Phenol (pure crystals)	10	grams.
Lactic acid (*syrup S C.* 1·21)	10	„
Glycerol	20	ml.
Distilled water	10	„

Dissolve the phenol in the water and add the other ingredients.

0·01 per cent. cotton-blue may be added for demonstrating the structures of organisms.

Technique

Wet preparations are prepared in the usual manner, using the lacto-phenol as a mountant Specimens must be handled very carefully and must not be " mixed " with the fluid as the delicate hyphae are very easily broken up.

Picric acid-nigrosin solution (Moulds)

1 per cent picric acid solution	1	part.
10 per cent. nigrosin solution (watery)	1	„

thicker portions purple-blue. The stain, which keeps well, provided it is kept in an airtight vessel, is best left for twenty-four hours before use.

Negri bodies stain bright red, cytoplasm of nerve cells stains purple-blue, nuclei and nucleoli of nerve cells stain deep blue, the stroma stain rose-pink. Nerve fibres stain deep-pink, the neural sheath does not stain. Any bacteria present stain an intense blue ; muscle fibres stain a brick-red and red blood cells copper colour.

Methods for Demonstrating Protozoa

Carnoy's fixative (for amoebae)

Absolute alcohol	6 vols.
Chloroform	3 „
Acetic acid (glacial)	1 vol.

Technique

Wet films are immersed in the solution for ten minutes, followed by washing and then immersion in absolute alcohol for ten minutes. The films are passed through 95, 70 and 50 per cent. alcohol, finally into distilled water for ten to twenty minutes before staining (see below).

Mercuric chloride fixative (for amoebae)

Saturated aqueous solution mercuric chloride	2 vols.
Absolute alcohol	1 vol.

Technique

Wet films are immersed in the solution for five to ten minutes, followed by washing in 50 per cent. alcohol. They are then flooded with Gram's iodine for two minutes and again washed in alcohol. Bring to water through varying strengths of alcohol before staining (see below).

Noller's method (for intestinal flagellates)

Fix moist films in a warm strong solution of mercuric chloride for from ten to thirty minutes and wash in gently running water for ten minutes. Flood the films with several

washings of N/saline solution, drain and immerse the slides in sterile serum (horse or beast) for five to fifteen minutes. Remove the slides and allow them to drain dry, wiping off the serum from the back of the slides. Fix films in absolute alcohol for ten to twenty minutes and allow to dry before staining (see below).

Giemsa's method (for intestinal flagellates)

Moist films are fixed in a solution of 1 volume of 95 per cent. alcohol mixed with 2 volumes of saturated aqueous solution of mercuric chloride for from one to twelve hours They are then washed for a few seconds only in water and flooded with Lugol's iodine (diluted by adding 3 ml. of Lugol's iodine to 100 ml. of 2 per cent. potassium iodide solution) followed by a washing with 0·5 per cent sodium thiosulphate solution. Wash in running water for five minutes and stain with diluted Giemsa's stain (as used in staining blood films) for one to ten hours. Wash, dehydrate, clear and mount.

Staining Protozoa

Iron-haematoxylin

(*a*) Haematoxylin	1	gram.
Absolute alcohol	100	ml.
(*b*) Liquor ferri perchloride	4	„
Hydrochloric acid (conc.)	1	„
Distilled water	100	„

The solutions are kept separately and equal parts mixed just prior to use.

Technique

After fixation and reducing, through various strengths of alcohol, to water, films are flooded with the solution and allowed to remain for from ten to twenty minutes. They are next placed, film uppermost, in a shallow dish through which a slow current of tap water flows and left for at least ten minutes. The slides are then drained, passed through 50, 75 per cent., and absolute alcohols, cleared in xylol and mounted in canada balsam.

Films may be counterstained by immersion, for thirty seconds, in Van Gieson's solution :

Saturated aqueous solution acid fuchsin	2 vols.
Saturated aqueous solution picric acid	100 „

just prior to dehydration, followed by immersion directly into absolute alcohol for a few moments only before clearing in xylol.

Mallory's method (for amoebae)

After fixation, films are stained with a saturated aqueous solution of thionin for five minutes followed by thorough washing in water, dehydration through varying strengths of alcohol and clearing in xylol, then mounted in canada balsam. The nuclei of amoebae appear a brownish-red.

Kofoid's modification of Donaldson's method (for amoebae)

Solution No. 1

Saturated solution of eosin in N/saline solution.

Solution No. 2

5 per cent. potassium iodide solution saturated with iodine.

(Equal parts of the solutions are mixed just prior to use.)

Technique

Place a small drop of the eosin-iodine solution on a slide and, close to it, a small drop of saline emulsion of the material to be examined. *Cover both drops with a cover-slip* and examine the area where the two drops intermingle.

Faecal material and certain intestinal yeasts become stained almost immediately. Protozoal cysts gradually become yellow (of varying shades) ; glycogen-filled vacuoles dark brown ; nuclei, particularly those of *E. coli* and *E. hystolitica*, are deeply stained with the iodine. The bulk of the material affords a pink background against which the protozoa are readily distinguished.

Panoptic method (combination of Leishman and Giemsa)

Technique

Without previous fixation, cover films with Leishman's stain and allow to stand for one minute, then dilute the stain on the slide as for blood-films and leave for ten minutes. Wash the films in water and then flood with diluted Giemsa's stain. Allow this to remain for from thirty minutes to twenty-four hours. Wash in water and differentiate with 1·1,000 solution of acetic acid until the stain just commences to diffuse into the acid solution Wash in water, pass through varying strengths of alcohol up to absolute, clear in xylol and mount in liquid paraffin.

Wet-Film method (for amoebae and flagellates)

(Army Pathological Services, Current Notes, No. 14, January 1945.) A M. A. Beemer (*verbatim*)

Solution "A"

Brilliant cresol blue	0·2 gram.
Sodium citrate	1·1 „
Sodium chloride	0·55 „
Mercury perchloride (saturated solution)	0·1 ml.
Water	100 „

Solution "B"

Eosin (water-soluble)	1 gram.
Water	100 ml.

Technique

Mix equal quantities of solutions "A" and "B" immediately before use and add one loopful of the mixture to a suspension of faeces on a microscope slide ; apply a coverslip and examine.

The stain gives the following appearances :

(*a*) Amoebae and flagellates that are not dead appear as clear, translucent, shiny, pale blue-green objects against a pink background ; they can be readily picked up with the lower power lens.

(*b*) Dead amoebae or flagellates take up the pink stain and the nuclei are usually quite distinct.

(*c*) Motility is unimpaired by the stain.

(*d*) By increasing the proportion of the solution "A" in the mixture, it can be shown that E. histolytica takes up particles of cresyl blue; this is contrary to what is usually recorded in textbooks. A good mixture to show this appearance is four parts of solution "A" to 1 part of solution "B". With this, vegetative forms of E. hystolytica look like blue monsters crawling across the field or charging clusters of dusty-pink erythrocytes. From these clusters the amoebae emerge stuffed with newly-ingested red cells.

The large species of E. histolytica seem relatively hardy for some have remained motile after sixteen hours on the bench at room temperature when stained in a wet film with equal parts of solutions "A" and "B".

Fixatives (for tissues)

Bouin's fluid

Picric acid (saturated aqueous solution)	75 ml.
Acetic acid (glacial)	5 „
Formalin	25 „

Formalin-dichromate solution

Potassium dichromate (1 per cent. aqueous)	50 ml.
Formalin	5 „
Distilled water	45 „

Formol-saline solution

Sodium chloride	8·5 grams.
Formalin	100 ml.
Distilled water	900 „

Kaiserling's solution

Potassium acetate	100 grams.
Glycerine	200 ml.
Water	1,000 „

Mann's fixative

Picric acid	1	gram.
Mercuric chloride	2·5	grams.
Distilled water	100	ml.

(when required for use add 15 ml. formalin).

Zenker-Formol

Mercuric chloride	5	grams.
Potassium dichromate	2·5	„
Sodium sulphate	1	gram.
Distilled water	100	ml.

(when required for use add 5 ml. formalin).

Zenker's solution

Mercuric chloride	5	grams.
Potassium dichromate	2·5	„
Sodium sulphate	1	gram.
Acetic acid (glacial)	5	ml.

Note.—When using fixatives containing mercuric chloride tissues should afterwards be immersed in 70 per cent alcohol containing a small amount of iodine which should be replaced as the alcohol becomes colourless during the process.

CHAPTER XXXI

THE MICROSCOPE

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Introduction

Before discussing the action and setting up of the microscope it is necessary to appreciate what is meant by the "Size of an object" and "Magnification". The eye may be considered to be of the simplified form shown in Fig. 48, its purpose being to form an image (I) of the object (O) upon the retina.

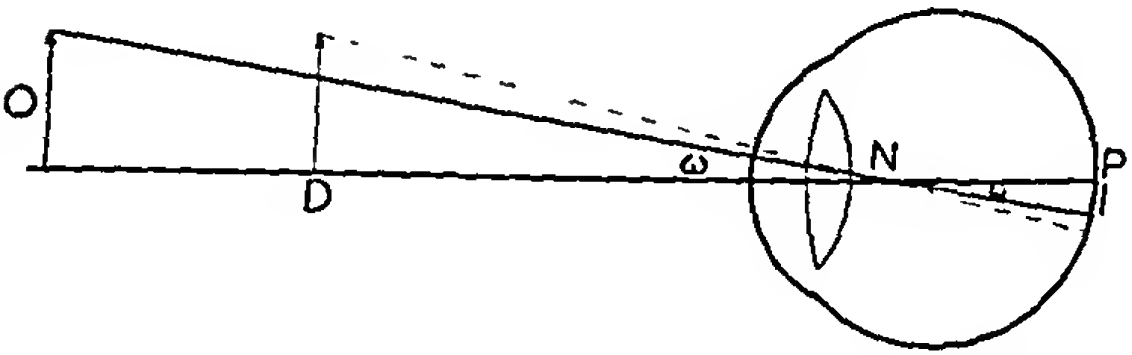


Fig 48

The size (I) of this image formed on the retina will be given by I=N.P tan. ω, that is, since N.P. is a fixed length, the size of the image is controlled by the angle ω which is the angle the object subtends to the eye. It follows therefore, that if two objects subtend the same angle at the eye, then they appear to be of the same size. If now the object is moved up to the eye then the angle it subtends will increase and the "size" of the object will increase. This process can only be continued until the object is at the Near Point (D) of the eye (10 in. or 25 cm. distant from the eye) since if the object is placed closer to the eye than the Near Point, the eye cannot accommodate itself to this and the image becomes indistinct. Magnification consists of increasing the angle an object subtends at the eye;

the simplest way of doing this being by a magnifying glass, which in its simplest form is a convex lens, as shown in Fig. 49.

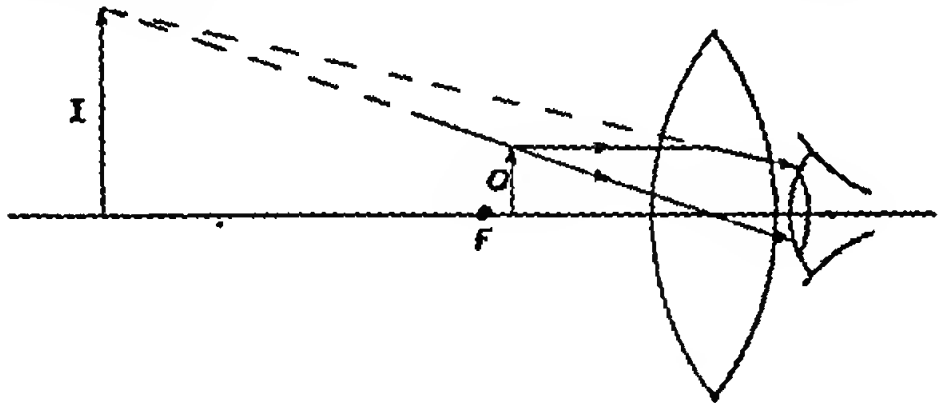


Fig 49

This lens forms a magnified virtual image (I) of the object (O) and then the eye placed close to the lens looks at this image It can be shown that the image will subtend the greatest angle at the eye when it is formed at the Near Point of the eye, the magnification then being $\frac{d}{f}+1$ where " d " is the least distance of distinct vision and " f " is the focal length.

This particular arrangement would cause the eye to be fully accommodated and result in tiredness, hence it is preferable

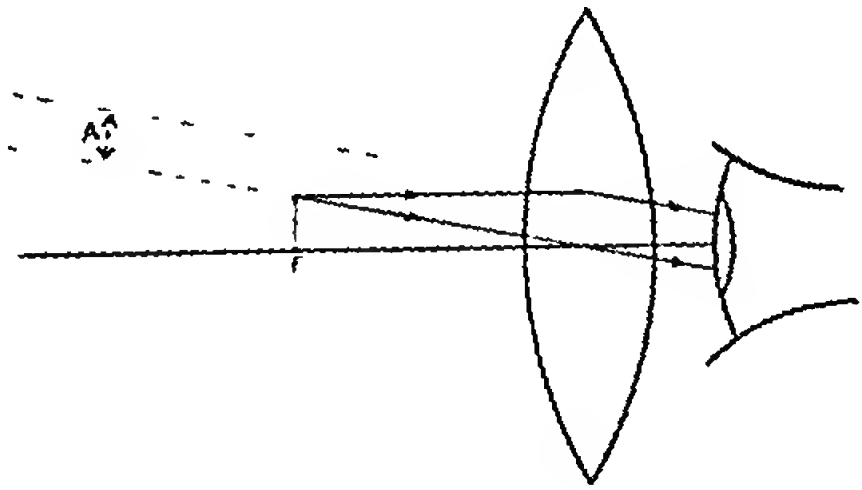


Fig 50

to arrange the position of the object so that the image is at infinity as in Fig. 50. The eye is then receiving parallel rays and is relaxed. In this case the magnification is reduced to $\frac{d}{f}$.

A single lens suffers from the following defects :

(*a*) Chromatic aberration, (*b*) Spherical aberration, (*c*) Coma, (*d*) Astigmatism, (*e*) Distortion, (*f*) Curvature of the field.

Of these the first two are axial defects, whilst the last four cause trouble with non-axial image points. By combining different glasses to make a compound lens some of the above may be reduced in magnitude. An example of this is the "Steinheil" magnifying glass shown in Fig. 51.

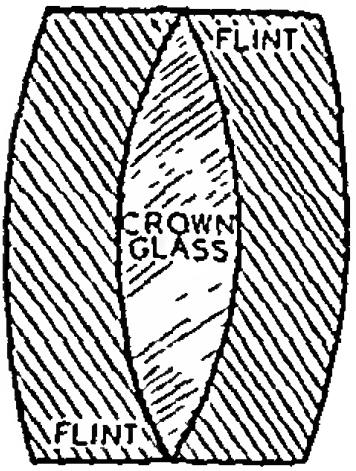


Fig. 51

Simple magnifying glasses, however, have a limit in that the object has to be at or within the focus, so that a magnification of $\times 10$ is about the limit. In order to obtain higher magnification a lens combination has to be used.

The compound microscope

The compound microscope consists of an object glass (O.G.), together with an eye-piece (E.P.) shown schematically in Fig. 52.

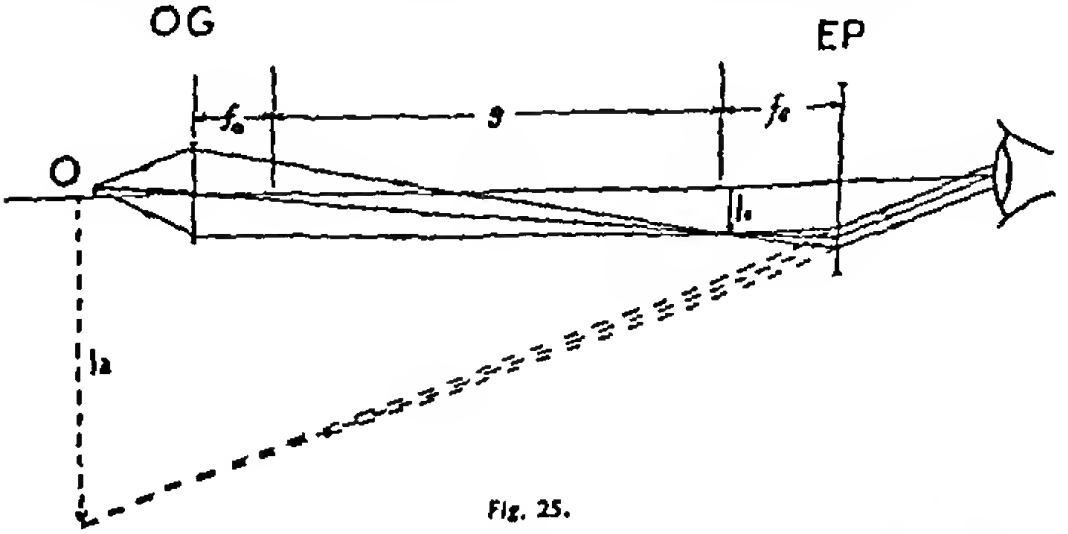


Fig. 25.

The purpose of the object-glass is to form an enlarged image (I_1) of the object (O), which image then serves as the object for the eye-piece. The eye-piece acting as a simple magnifying glass, forms a magnified image (I_2) which is "seen" by the eye. The magnification produced by the compound microscope is the product of the magnification given by the

object-glass and the magnification of the eye-piece, and it can be shown that the magnification is $\frac{g}{fo} \times \frac{d}{fe}$ where the symbols have the meanings given on page 286.

The objective

If the intermediate image (I_1) in Fig. 52 is of poor definition, then any magnification produced by the eye-piece will only

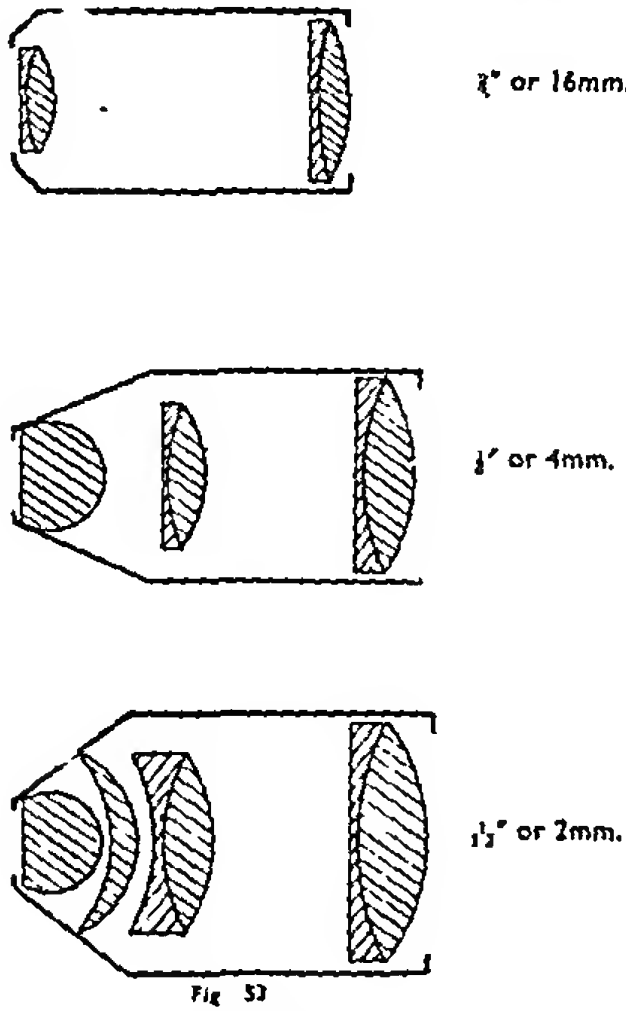


Fig 53

serve to magnify the defects. It is highly important therefore that the objective shall produce as good an image as possible. By employing several lenses made from different types of glass and suitably spaced, it is possible to obtain an objective which gives a really well-defined image. Typical objectives are shown schematically in Fig. 53.

Experience has shown that the above meet the usual requirements. A recent introduction is the 3·75 mm. oil immersion Fluorite objective which has the high numerical aperture of 0·95, together with a large depth of focus and is particularly useful for examining blood films for differential counting and films of sputum for M. tuberculosis.

A *Monochromatic objective* is one which will only give a well defined image with light of the single colour (wave length) for which that objective has been corrected; consequently its use is restricted.

An *Achromatic objective* has been corrected for two wavelengths and can be used over a limited range of colours.

An *Apochromatic objective* has a much more complicated structure and has been corrected for three wavelengths; it can be used over the whole range of colours and so is particularly suitable for use with stained specimens.

Focal length

The Focal Length is an optical constant of the lens-system and is measured from certain points inside the lens-system. Of more practical importance is the working distance, which is the distance of the object plane from the front surface of the first lens of the objective.

Depth of focus

The Depth of Focus is the thickness of the layer within the boundaries of which all points appear to be sharply in focus. The thickness of this layer is *very nearly* inversely proportional to the square of the numerical aperture: hence it follows that an objective with a large N.A. will have a small depth of focus.

Numerical aperture

The Numerical Aperture ("N.A.") is the measure of the ability of a perfect objective to reveal detail. Optical theory shows that the smallest distance between two points in an object just resolved is given by:

$$h = \frac{0 \cdot 61 \lambda}{n \sin. U}$$ (see list of symbols on page 286).

The numerical aperture (N.A.) is the denominator of the above expression (i.e n sin. U). From this we see that the larger the angle (U) of the cone of rays which enter the objective,

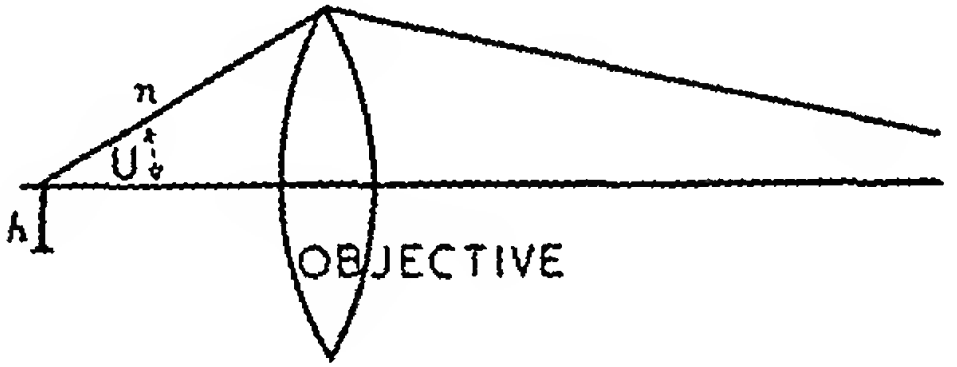


Fig 54.

the greater the numerical aperture and resolving power (see Fig. 54). The manner in which the cone of rays entering the objective is influenced by the medium between the object and the objective is illustrated in Figs. 55(a) and 55(b)

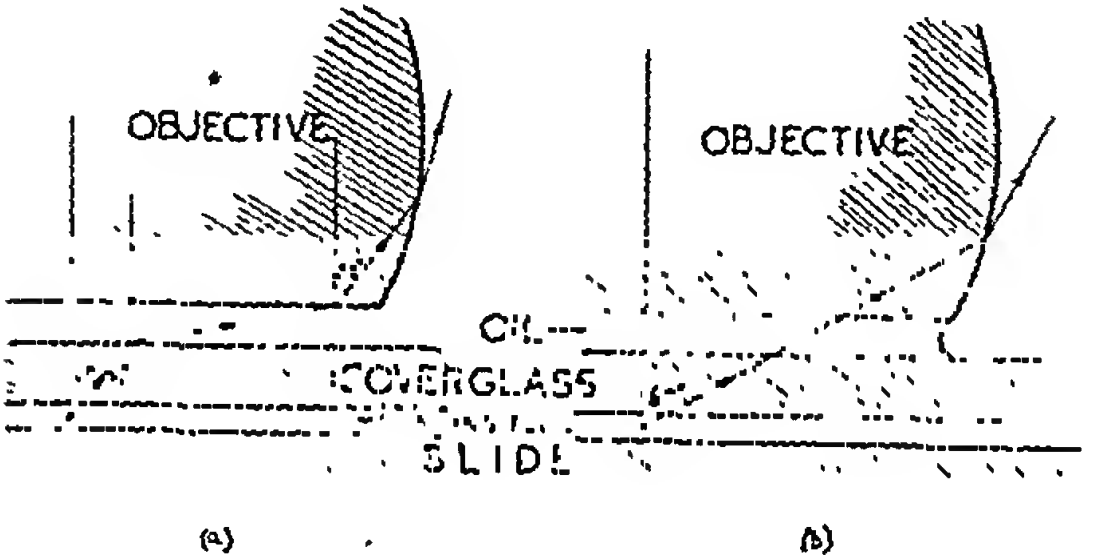


Fig 55.

In Fig. 55a, because of the air gap, the maximum practical angle for the cone of light in the objective is 39°, whereas in Fig. 55b we see that the introduction of oil of the same refractive index as glass allows a cone of 60° angle to enter the objective with consequently a larger resolving power. It must be noticed that if a $\frac{1}{12}$th objective is used without oil, then the N.A. will

not be 1·3, but about 0·9, a value which can be obtained with a much cheaper objective.

Resolving power

The Resolving Power of a microscope may be defined as : $\frac{I}{h} = \frac{N.A.}{0{\cdot}61\lambda}$ and is the reciprocal of the separation of the smallest detail just resolved. Having obtained the necessary resolution to separate the smallest detail then we must employ sufficient magnification to reveal this detail to the eye. Any further magnification is "spurious" or "empty" magnification. The useful magnification is generally accepted to be 1,000 × the N.A.

i.e. if the N.A. is 1·3, then the limit of useful magnification is 1,300 ×.

The difference between magnification and resolution must be appreciated ; magnification consists of increasing the apparent size of an object, whereas resolution is the term applied to the ability of the objective to form separate distinct image-points of the fine structure of the object.

When examining objects which have appreciable thickness, it is frequently advisable to use the lowest objective which will resolve the necessary details, since this will give the greatest depth of focus.

Referring to Fig. 55 we see that with "dry" objectives the cover-glass affects the path of the light from the object to the objective. Objectives are usually designed for use with cover-glasses whose thickness is 0·17 mm. and a tube length of 160 mm. If the cover-glass is too thin then spherical aberration is introduced, which can be corrected by increasing the tube-length. The reverse is true if the cover-glass is too thick. The tube-length should not be increased to give extra magnification, since this only upsets the corrections which the designer of the objective has been at such pains to achieve.

The Eye-piece (ocular)

Eye-pieces are usually of three types, the **Huygenian, Compensating,** and **Ramsden.**

The most frequently-used type of eye-piece is the Huygenian

which gives well-corrected images with medium- and low-power objectives. With high-power objectives, however, the marginal parts of the image show some colour fringes. The compensating eye-piece has been designed to correct these oblique chromatic errors. This type of eye-piece is particularly useful with apochromatic objectives. A further advantage is that

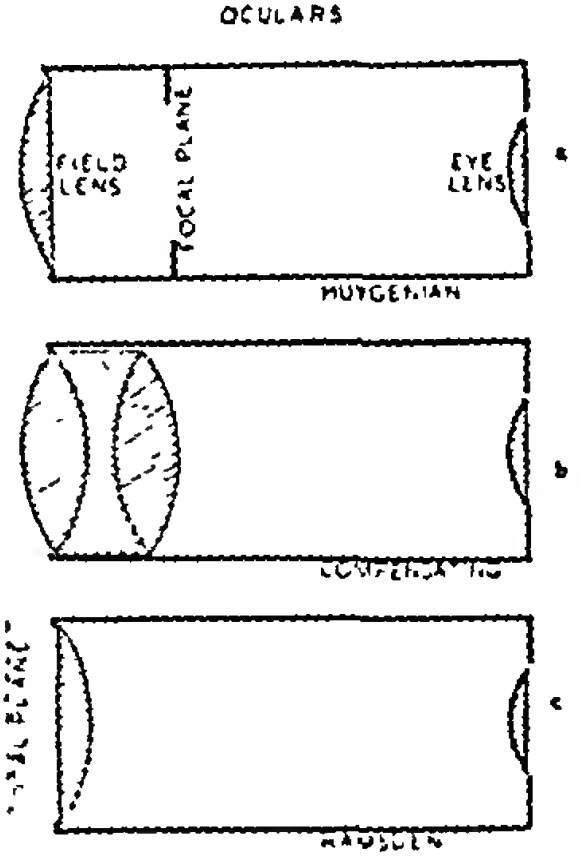


Fig 56

a flatter field is given by the compensating type of eye-piece. The Ramsden eye-piece differs from the other two in that its focal plane is without the eye-piece. This makes it particularly suitable for use as a micrometer eye-piece

The Stand and Mechanical Parts of the Microscope

The stand and mechanical parts of a typical microscope are illustrated in Fig. 57 (page 272)

Dealing with the various parts from the base upwards, we have

The Foot. This may be of the horse-shoe or tripod type,

but whichever is chosen it should be sufficiently large, both in size and weight to ensure stability.

The Limb. The limb carries the body of the instrument which articulates with the body-tube by means of the coarse adjustment.

Fine Adjustment. This produces the extremely slow motion necessary when focussing the object, and moves the body relative to the limb. The fine adjustment must be sufficiently well-made to fulfil the conditions of providing motion which will comply with the depth of focus requirements which, in the case of the $\frac{1}{12}$th-in. objective is in the order of 0·0004 mm.

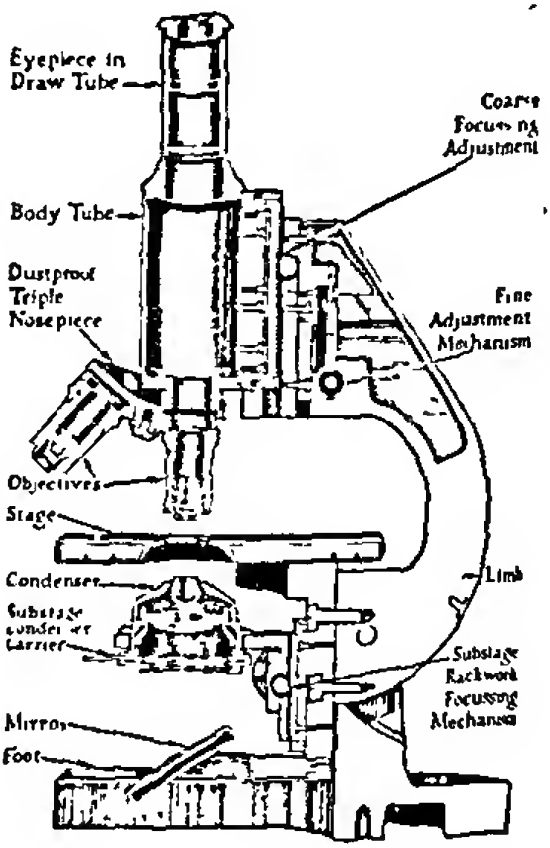


Fig 57

(From *The Microscope, Its Theory and Applications*, by J. H. Wredden J. & A. Churchill Ltd)

The Stage is the rigid platform upon which the object to be examined is placed. Frequently it has fitted on it or better, built into it, a mechanical stage by which the object may be moved.

Mirror. The mirror is carried by the tail-piece which upper end is fitted into the substage assembly. This

substage assembly should be fitted with coarse and fine focusing adjustment, but many microscopes are fitted with coarse adjustment only at this point.

Substage Condenser. It should be possible to centre the substage condenser by means of special adjusting screws. The condenser is fitted with an iris diaphragm.

Nose-piece. This may be single, double, triple and, on some instruments, provision for four or even more objectives is made. Objectives in general use are kept permanently attached to the multi-nose pieces, it being necessary only to revolve the nose-piece to bring the objective required into use.

The Body-tube (sometimes referred to as the barrel) The upper part of this carries the draw-tube into which fits the eye-piece. Many modern microscopes are of "fixed tube length" and no draw tube is fitted; this means that no correction can be made for cover-glass thickness.

The Draw-tube. This is for adjustment of tube-length to correct the instrument for cover-glass thicknesses.

Illumination of the object

Four requirements must be fulfilled in illuminating the object:

(1) The illumination must be even; (2) the cone of light from the substage condenser must be large enough to fill the objective (i.e. the N.A. of the substage condenser must be equal to that of the objective; (3) only that portion of the object which is to be observed should be illuminated; (4) the amount of scattered light must be reduced to a minimum.

Possibly the most convenient source of light is a modern opal-type-bulb electric lamp, or, alternatively, a clear lamp with a piece of opal glass in front of it Such a lamp should be housed in a fitting carrying an iris diaphragm and slots for colour-filters (see Fig 58)

The concave mirror is only for illuminating the object when low-power objectives are being used In such cases the cone of light from the mirror is sufficient to fill the objective, the position of the lamp being adjusted until a sharp image of the lamp is formed in the plane of the objective (a pencil held against

the lamp will be found to assist in determining when correct adjustment is achieved).

The substage condenser is used with higher-power objectives ; the *plane* side of the mirror being used in this case. The usual

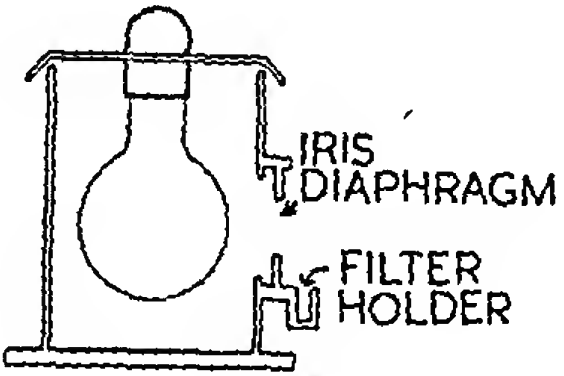


Fig. 58

type of condenser is the Abbé which, however, is not corrected for chromatic or spherical aberration. Consequently a poor image of the source of light is formed in the plane of the object, which results in somewhat uneven illumination and much scattered light. A better type of condenser is the achromatic

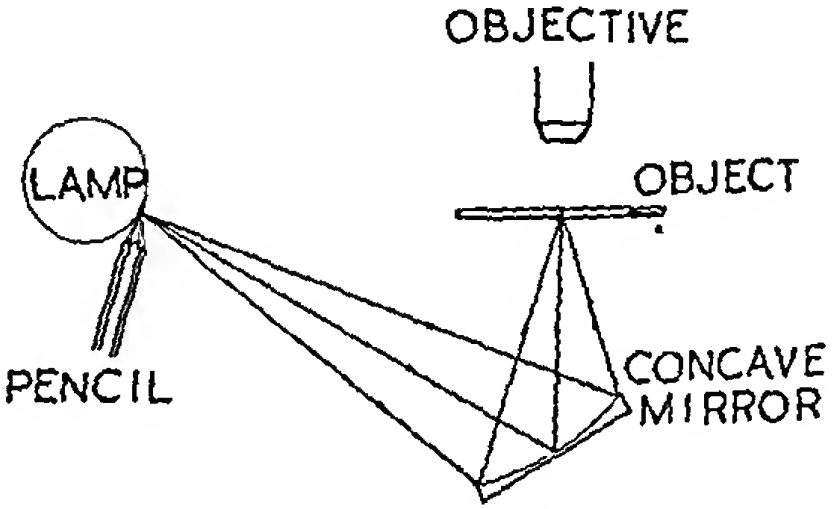
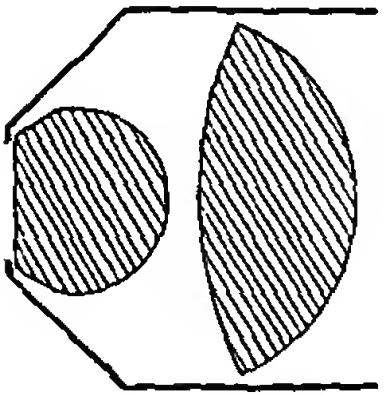


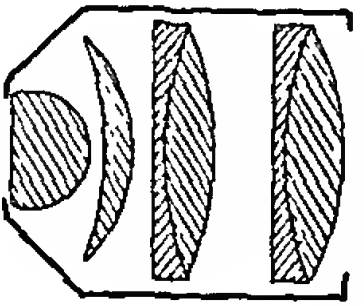
Fig 59.

condenser, since it is corrected for spherical and chromatic aberration. This condenser should be used with the $\frac{1}{12}$th " oil immersion " objective if full value of the N.A. is to be obtained. It must be stressed that all condensers if used " dry " cannot have a greater N.A. than 1·0, the same reasons

holding as for objectives Therefore, if an oil immersion objective is in use, the condenser should be oiled to the slide.



Abbé Condenser



Achromatic Condenser

Fig 60

When the source of light is a large opal bulb, then the substage condenser is used to form an image of the source of light in the plane of the object as shown in Fig 61.

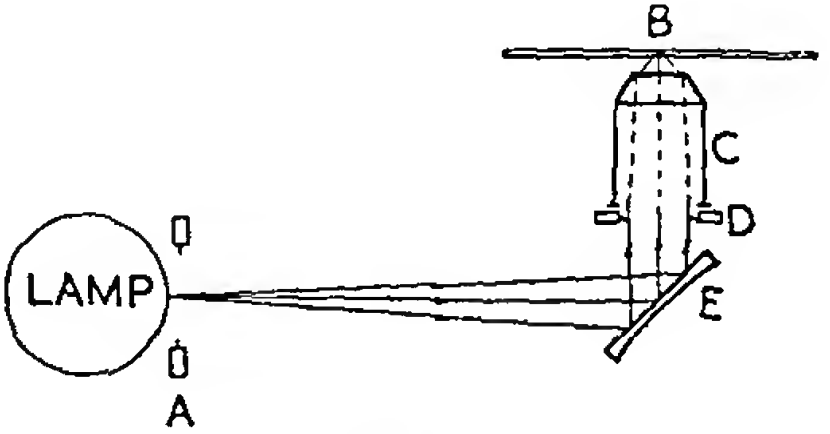


Fig 61

A—Lamp iris
B—Object
C—Substage condenser
D—Substage iris
E—Mirror

The substage iris regulates the angular width of the cone of rays illuminating the object, whilst the lamp iris limits the area of the object which is illuminated. When an opal lamp does not give sufficient intensity of illumination and a high intensity source such as a " Point-o-lite " lamp or arc lamp has to be used, then the size of the source has to be effectively increased by the use of a condenser which we will call the lamp condenser. This arrangement is shown in Fig. 62.

The lamp condenser (A) should be achromatic and its focal length chosen so that it gives of the source an enlarged image which falls on and completely fills the aperture of the substage iris. The substage condenser is then racked up or down until an image of the lamp condenser is formed in the plane

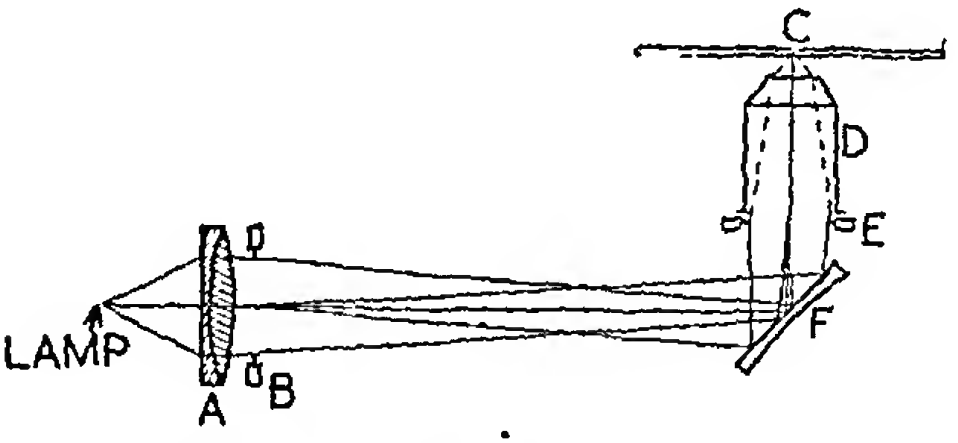


Fig. 62

A—Lamp Condenser
B—Lamp iris
C—Object
D—Substage condenser
E—Substage iris
F—Mirror

of the object—again a pencil held against the lamp condenser will assist in determining this. As before, the substage iris *regulates the angle of the cone of rays, whilst the lamp iris* controls the area of the object illuminated.

When opaque objects have to be examined the above methods can no longer be used.

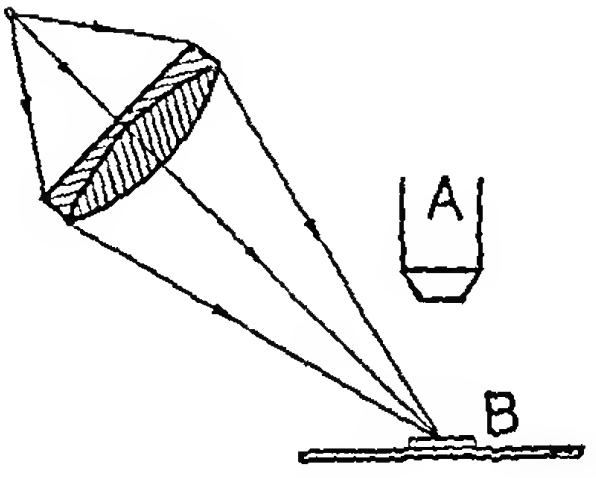


Fig 63(a).

A—Microscope
B—Object

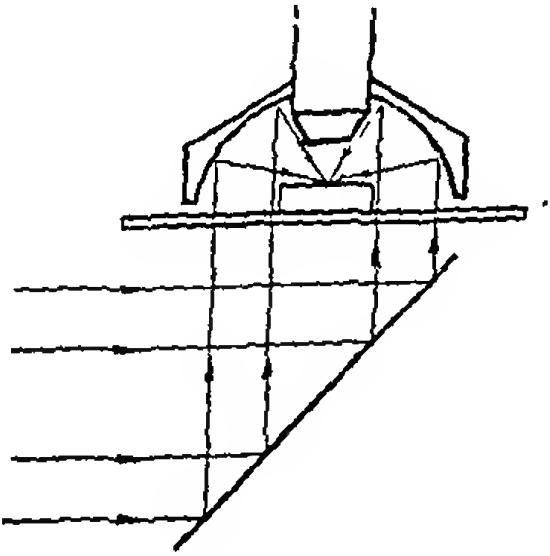


Fig 63(b)

For low-power objectives simple oblique illumination such as is illustrated in Fig. 63a is often satisfactory. With objectives of slightly higher power, say $\frac{1}{3}$ in., better illumination is obtained with a paraboloid reflector fitted to the nose of the objective as in Fig. 63b.

In both of these methods the directly reflected light does not enter the objective and the image which is seen is a " shadow

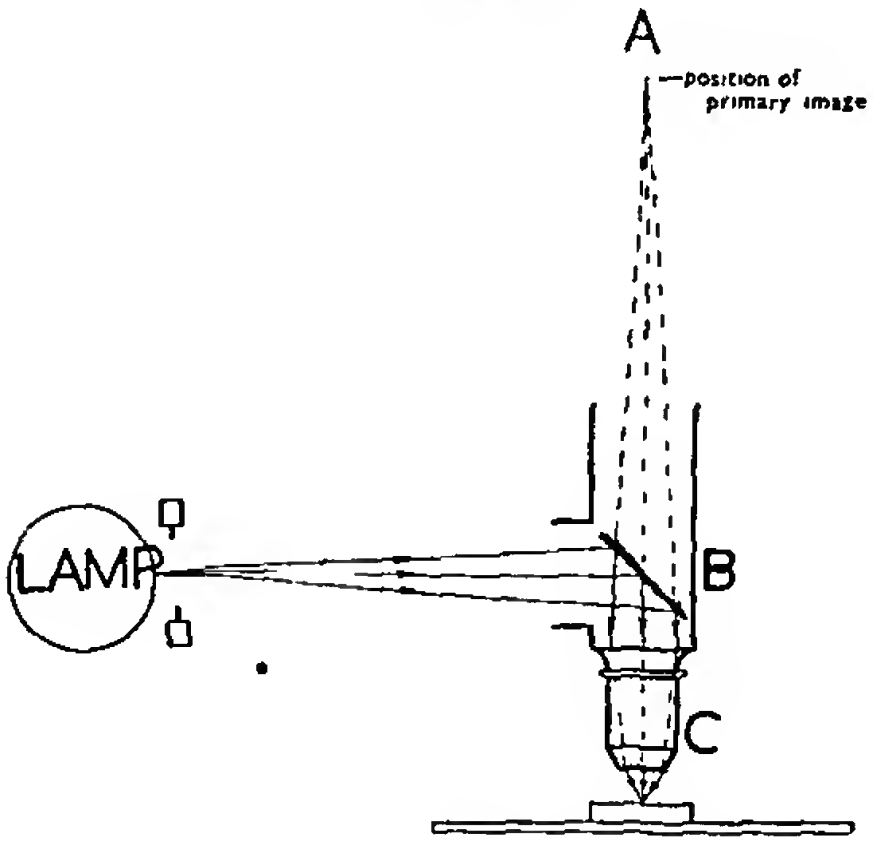


Fig 64(a).
Distance of Lamp to B is equal to distance of A from B

relief " of the surface of the object. Such a type of illumination is really " Darkground illumination " with which we will deal more fully later. Contrasting with this oblique type of illumination is " Vertical illumination ", in which the light is reflected down through the lens on to the object The vertical illuminator in its simplest form is a plane parallel glass plate held in a suitable mount which screws into the body tube just above the objective. Its action is illustrated in Fig 64a. Sometimes the plane glass reflector is replaced by a prism which projects over a part of the lens. The prism gives a brighter illumination of the object and less scattered light so that the

contrast is greater, but, of course, since the aperture is restricted the resolution will be decreased. As with the illumination of transparent objects, a well-defined image of the source must be formed in the plane of the object. Since the objective is acting as the condenser, it follows that the source of light, or the effective source of light, must be at the same distance from the

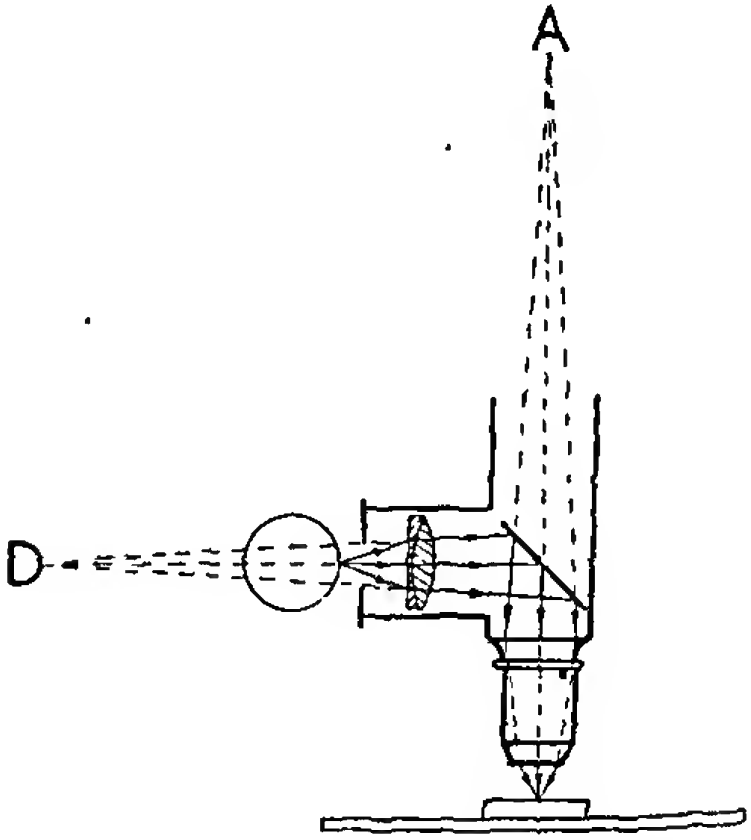


Fig 64(b).

A—Primary image D—Effective source of light

objective as the primary image A. The methods of achieving this are illustrated in Figs. 64a and 64c which represent those usually adopted. Fig. 64b shows the method adopted when the microscope has "built-in" illumination.

In the case represented by Fig. 64c, using a "Point-o-lite" source of light, the lamp condenser forms an enlarged image of the source completely filling the aperture of the field iris, thus this image acts as the source of light. This condenser must be so designed that it gives the necessary image of the source when its distance from the field lens is such that the latter produces an image of the lamp iris on and completely filling

the rear surface of the objective. Variation of the lamp iris varies the aperture of the objective, whilst variation of the field iris controls the area of the object illuminated.

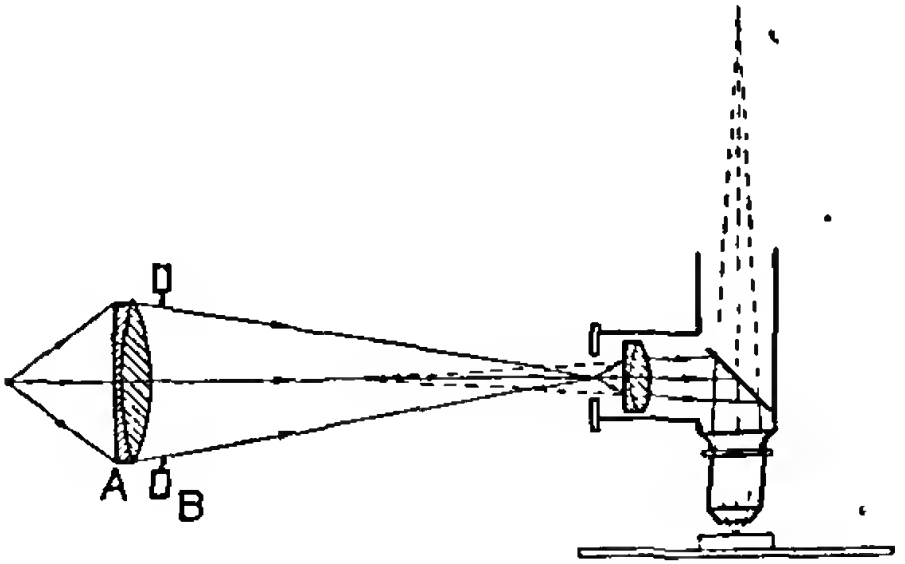


Fig 64(c)

Darkground illumination

We must now return to darkground illumination in which the brightly illuminated object is seen against a dark background. Just as it is easy to see spiders' webs brightly

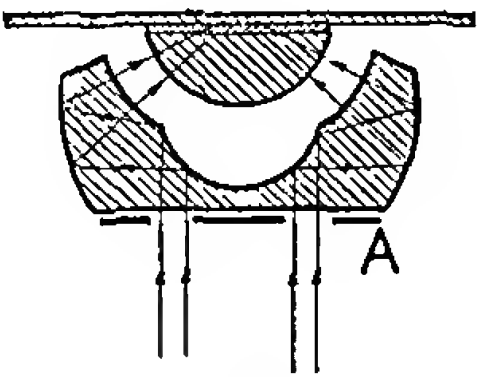


Fig 65.

illuminated by sunlight against the dark background of a bush, so the flagella of certain bacteria have been made visible by darkground illumination. The drawback to this type of illumination is that it produces bad diffraction effects which cause confusion in interpreting the image. The Beck focusing darkground illuminator is illustrated in Fig. 65 This

condenser has the advantage that it can be adjusted for variations of slide thickness.

The light strikes the object as a hollow cone and in order that the angle of this cone shall be large enough to be without the objective, the dark ground condenser must be oiled to the slide. *It is impracticable to make the dark inner cone of greater* N.A. than about 1·0, so if an oil-immersion lens of N.A. 1·2 were used, some direct light would enter the objective; in such a case a funnel-stop is screwed into the back of the objective, reducing the N.A. to 0·95. The arrangement for obtaining satisfactory illumination is as in Fig. 62, it being necessary to use a source of high-intensity light such as a "Point-o-lite" lamp. When nearly adjusted a ring of light will be seen on the slide, this is first centred by adjusting the condenser and then reduced until it is as small as possible.

Routine Method for Correct Setting-up of the Microscope

If the full value of the microscope is to be developed, then as we have seen, attention must be given to the adjustments, and below is given a routine which will ensure that these are made correctly. In the case of some microscopes, especially *those with built-in illuminating systems, the maker's literature* usually gives the necessary information.

(*a*) Arrange the microscope and source of light in convenient positions, the distance of the lamp being suitable for the particular lamp-condenser (if this is used).

(*b*) With all optical parts removed, tilt the mirror (plane side if the substage condenser is being used) until the light is coming directly up the tube.

(*c*) Place the object on the stage and, using a low-power objective ($\frac{2}{3}$ in) and a medium-power eye-piece, focus the object. Adjust the tube length to the correct value for the particular cover-glass thickness.

(*d*) Replace the substage condenser and adjust it until the lamp iris diaphragm is focussed on the object (a pencil held in front of the lamp will help in this). With the use of an oil-immersion objective, it is advisable to place a drop of immersion oil on the top surface of the condenser so as to oil the condenser

to the slide; this is essential if maximum resolution is to be obtained.

(*e*) Remove the eye-piece, and looking down the tube into the back surface of the objective , close the substage iris until the edge of the iris is seen inside the objective. The condenser is now centred by means of the centering screws, until the image of the hole in the diaphragm is concentric with the objective.

(*f*) With the eye-piece still removed, open the substage iris diaphragm until the objective is almost completely filled with light

(*g*) Replacing the eye-piece, adjust the lamp-iris until that portion of the object under observation is illuminated After this it is advisable to again remove the eye-piece and verify that the aperture has not been reduced.

(*h*) When the eye-piece is replaced, the instrument is ready for use.

(*j*) After changing the objective for one of higher power, repeat (*f*), (*g*) and (*h*)

If the intensity of illumination is too great, the best method of reducing it is to place a neutral tint filter of suitable density in front of the lamp Frequently greater contrast can be obtained by using a filter of colour complementary to the colour of the object, thus if the object is red and a green filter is used, then the red will be darkened.

A colour filter helps in resolving fine detail since even though the objective be corrected for several wavelengths better definition is obtained if the light is restricted to a single wavelength, also if the filter is green-blue or blue, the shorter wavelength gives increased resolving power.

We have made reference to immersion oil and its use, this is a special cedar-oil whose refractive index is 1·515. When removing the oil after use, considerable care is required in order not to damage the hyper-hemispherical front lens of the objective. This lens is usually only held in its mount with cement and undue pressure may result in displacement of the lens. The cedar-oil is best removed by wiping gently with a piece of silk dipped in benzene. Cedar-oil tends to thicken when kept, its refractive index becoming more nearly that of the cover-glass, but when it becomes very sticky it should not be

used, since the front lens may tend to stick to the slide. It has been shown that the immersion fluid is used so that there is no air-gap between the condenser and slide, and the cover-glass and objective ; the immersion oil making an optically uniform medium. If the specimen is " dry " mounted, i.e. there is air between the slide and cover-glass the advantages of a uniform medium are lost. Therefore, if the use of an oil immersion lens is contemplated the specimen must be mounted in canada balsam or other medium of suitable refractive index. Canada balsam is a pure form of turpentine and is generally used diluted with xylol. On being kept in a warm place for twenty-four hours or so the balsam hardens ; its refractive index then being about 1·52. (*Note.*—Where film preparations are examined the immersion oil may be placed directly on the film and the question of air gaps under the cover-glass does not arise.)

Interpretation of the image

It is frequently assumed that the image seen in the microscope is the image of a single plane. This is not so, however ; what is seen is the sharp image of a single plane superimposed upon which is the out-of-focus images of the planes above and below the true object plane.

The image is further complicated by the reflection, refraction and defraction of light around the object so that the appearance can be very deceptive. An "*artificial*" *object which is* representative of many cases which arise is a suspension of fat globules in water (the refractive index of the fat is usually higher than that of the water). When the microscope is focussed on the remote side of a globule one sees a disc with a faint dark ring at the edge, on racking upwards the appearance changes to a spot of light in the centre with a dark ring at the edge.

When *oblique or dark-ground illumination* is used the diffraction effects are much more pronounced and unless care is taken, a structure is attributed to the object, when no such structure exists. No general rule can be given for determining whether or not what is seen actually exists. If it is possible,

the relative phases of the direct (A) and diffracted (B) beams of light so that in effect the diffracted light becomes enhanced. The method is illustrated in Fig. 67 and consists of replacing the substage condenser iris by a diaphragm (F) in the form of an annulus. The phase plate (C) is then placed in the back focal plane of the objective. Typical results are shown in Fig. 68.

For further information regarding phase-contrast microscopy, see the references below.*

Fluorescent Microscopy

In fluorescent microscopy, which has been used for the detection of M. tuberculosis, the specimen is stained with a fluorescent dye and is then illuminated with ultra-violet light. Under these conditions the object fluoresces brightly whilst the background is dark. The normal microscope is used, the lamp being replaced by, say, a mercury-vapour lamp and a filter to cut out the visible light. Since the silver of the substage mirror absorbs the ultra-violet rays, it is replaced by an aluminium mirror. A yellow filter placed in the eye-piece allows the yellow light from the bacilli which are fluorescing to pass, but cuts out any other light.

Ultra-violet Microscopy

When dealing with the resolving power of the microscope we

Phase-contrast

Most microscopic objects and especially biological ones have different refractive indices in the various parts of their structure. This variation in refractive index results in the

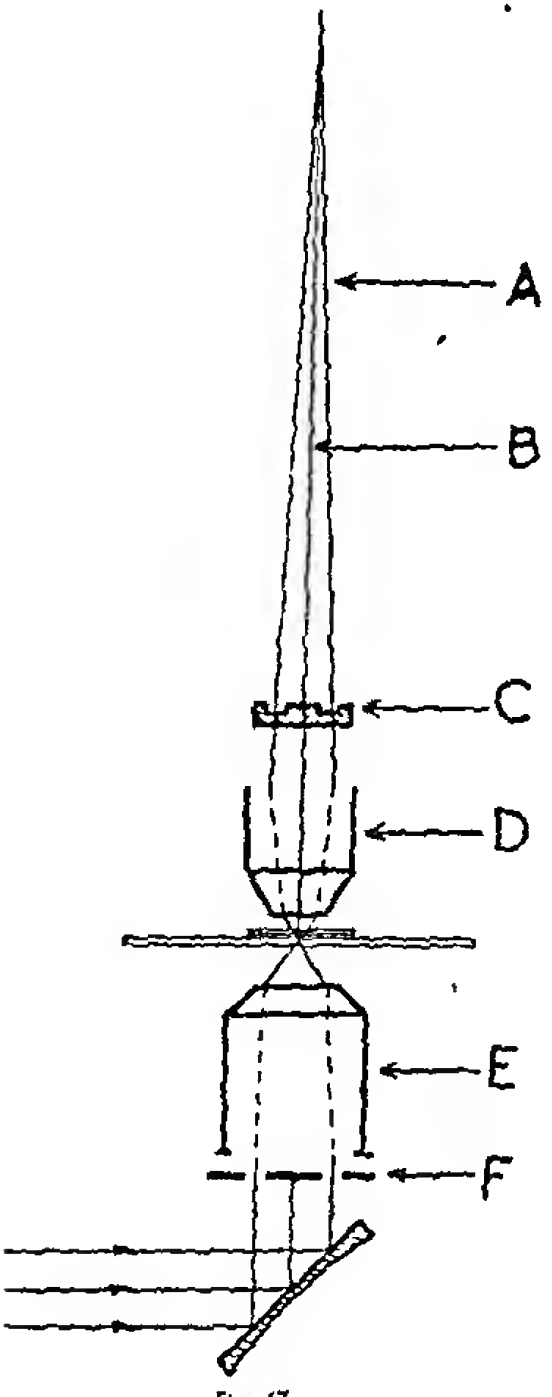


Fig 67

diffraction of light. Unless the difference of refractive index is very great, the amount of diffracted light is small, so that its effect is lost. The method of phase-contrast is to alter

the relative phases of the direct (A) and diffracted (B) beams of light so that in effect the diffracted light becomes enhanced. The method is illustrated in Fig 67 and consists of replacing the substage condenser iris by a diaphragm (F) in the form of an annulus. The phase plate (C) is then placed in the back focal plane of the objective. Typical results are shown in Fig. 68.

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Ultra-violet Microscopy

When dealing with the resolving power of the microscope we saw that the resolving power is $\frac{1}{h} = \frac{\text{N.A.}}{0{\cdot}61\lambda}$, thus the smaller the wavelength (λ) of light we use, the greater will be the resolving power and the use of ultra-violet light of wavelengths $0{\cdot}4\mu$ to $0{\cdot}2\mu$ is indicated. Since the U.V. is not visible it means that photography has to be used. In Fig. 69 are photographs which show the additional resolution achieved by the use of U V.

Further interesting results are shown in Fig. 70 where we see how the different parts of the retina of an owl have absorbed differentially the various wavelengths. By correlating this differential absorption with known absorption spectra a way is open to identification of the cell constituents.

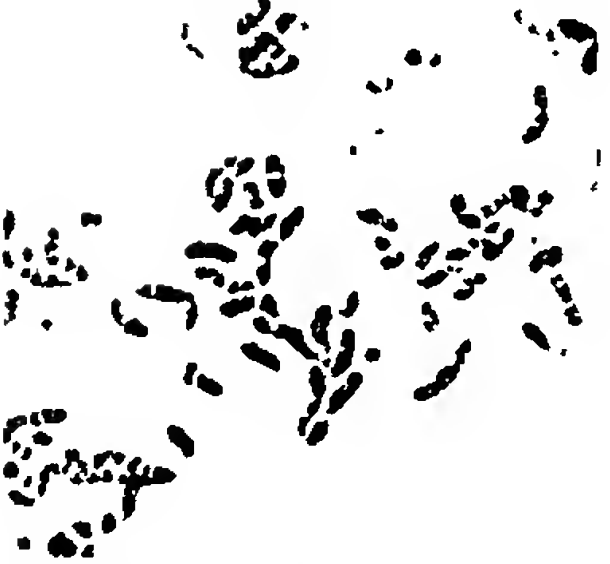
* References Burch and Stock. *J Sci. Inst.*, 19, No 5, 71-5 (1942).
Linfoot, E H *Nature*, 155, 76 (1945)

Conclusion

The above survey of the microscope and its use has of necessity, been brief. For further information the reader is referred to *Practical Microscopy* (Martin and Johnson).

Below is given a list of the symbols which have been used in this chapter, together with their meanings.

d—Least distance of distinct vision, i.e. distance of Near Point from eye.
f—Focal length of a lens or lens-system.
g—Optical tube length.
h—Smallest distance between two points of an object which give separate image points
n—Refractive index of medium.
λ—Wave-length of light.
ω—Angle subtended by object at eye.
F—Principal focus of lens or lens-system.
I—Image and/or size of image.
O—Object and/or size of object.
U—Semi-angle of cone of rays entering a lens or lens-system.
N.A.—Numerical Aperture.



(1) Positive phase contrast Large amplitude Phase difference $+\frac{1}{4}\lambda$

(2) Positive phase contrast Small amplitude Phase difference $+\frac{1}{4}\lambda$

(3) Transmitted light Narrow cone Appearance with substage condenser nearly closed

Fig 68

Caryophanum latum Objective 4 mm 375 ×

(E W Taylor *Proc Roy Soc* Vol 190)

(4) Negative phase contrast. Large amplitude. Phase difference $-\frac{1}{4}\lambda$.

(5) Negative phase contrast Small amplitude. Phase difference $-\frac{1}{4}\lambda$

Fig 68

Caryophanum latum Objective 4 mm 375×

(E W. Taylor. *Proc Roy Soc* Vol 190)

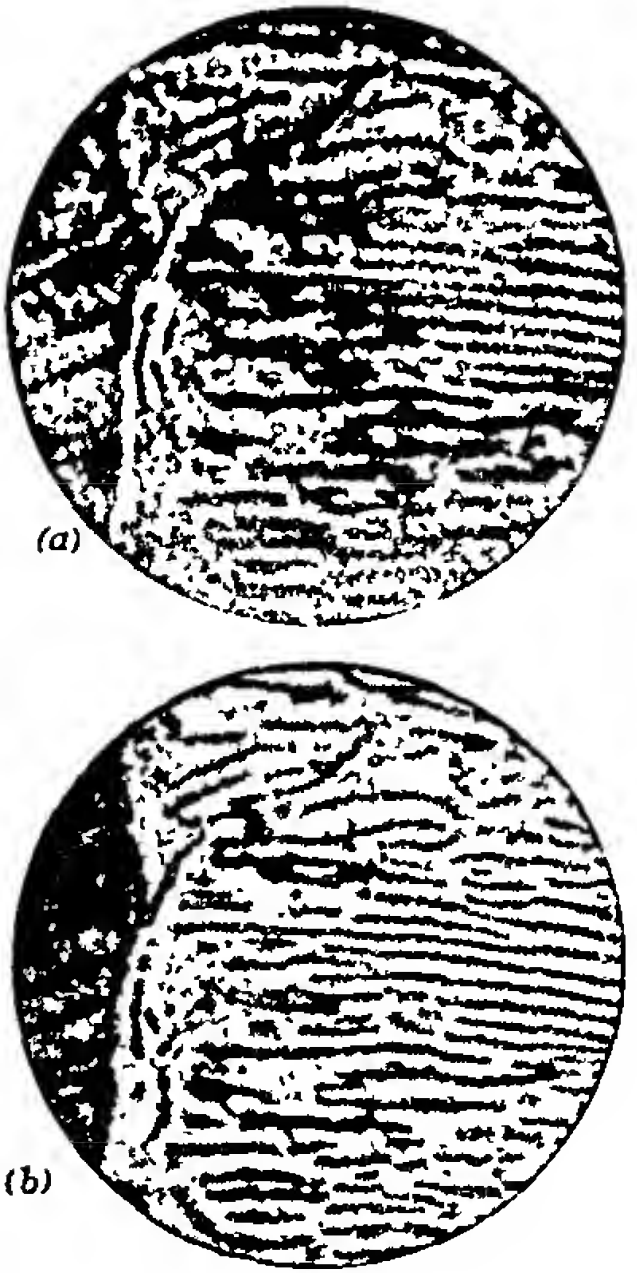
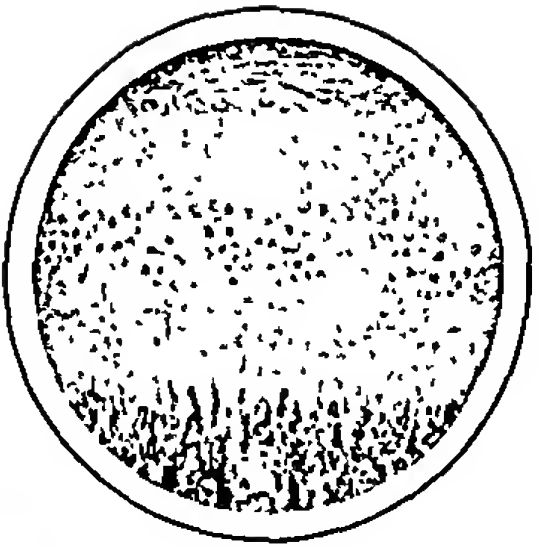


Fig 69

COMPARISON PHOTOMICROGRAPHS (taken in visual and ultra-violet illumination) showing increased resolution due to use of shorter wave length light The specimen is a 0 8 per cent carbon steel

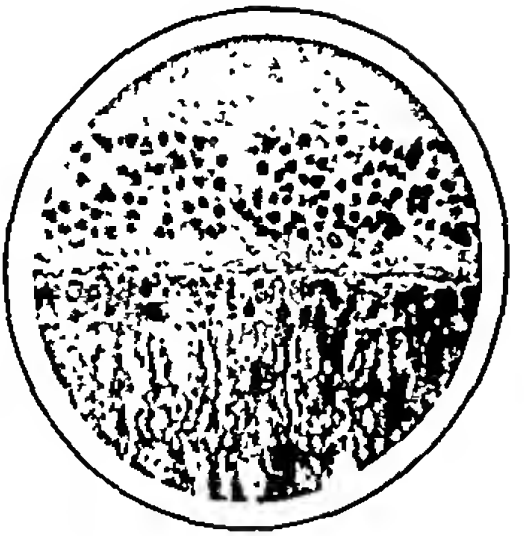
(a) Objective 2 mm Apochromat		(b) Objective 1 7 mm Monochromat	
Numerical aperture	— 1 30	Numerical aperture	1 25
Magnification	— 4 000	Magnification	4 000
Wave-length of light	0 55μ	Wave-length of light	0 275μ

(From *Practical Microscopy* by L C Martin and B K Johnson Blackie & Son)



λ—4500A

λ—2749A



λ—2313A

Fig 70

Showing Differential Absorption Effects when using various Ultra-Violet Wavelengths
(Specimen Retina of owl (unstained) Mag 450 ×)
(From *Practical Optics* by B K Johnson Hatton Press Ltd)

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